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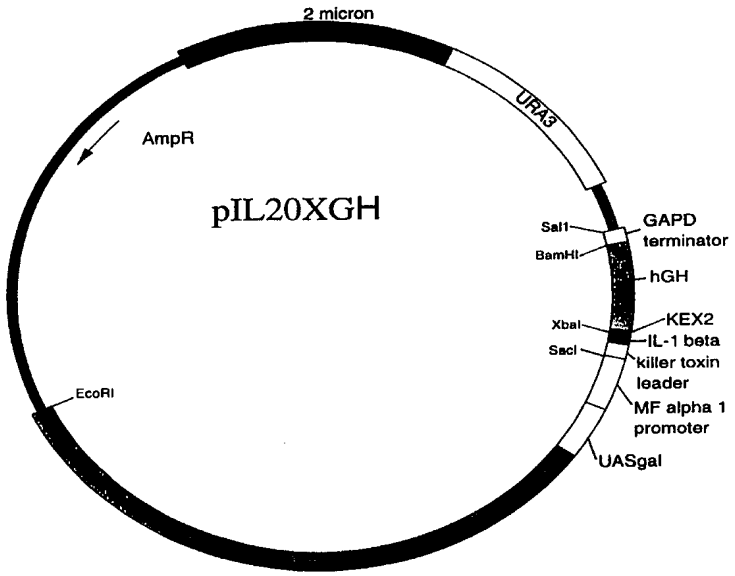
(54) Title: PROCESS FOR PREPARING RECOMBINANT PROTEINS USING HIGHLY EFFICIENT EXPRESSION VECTOR FROM *SACCHAROMYCES CEREVISIAE*



(57) Abstract

This invention concerns a method of producing human granulocyte colony-stimulating factor (hGCSF) and human growth hormone (hGH) from yeast by using recombinant DNA technology. More specifically, this invention relates to a method of producing hGCSF or hGH by using yeast expression vector which contains: hybrid promoter comprising promoters of two different yeast-derived genes, yeast killer toxin leader peptide, and amino terminus of IL-1β. In addition, this invention relates to a method of producing hGCSF by using expression vector which contains promoter and secretion signal of HSP 150.

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Title of Invention

Process for preparing recombinant proteins using highly efficient expression vector from *Saccharomyces cerevisiae*

Background of Invention

The present invention relates to a process for preparing recombinant proteins from yeast by using recombinant DNA technology. More particularly, the present invention relates to a process for preparing recombinant proteins by using yeast expression vectors which consist of hybrid promoter consisting of two kinds of yeast inducible promoters and secretory signal consisting of yeast killer toxin and amino terminal of mature interleukin 1ß(IL-1ß).

In addition, the present invention relates to a process for preparing hGCSF from yeast by using expression vector made up of promoter and secretory signal of yeast heat shock protein 150.

In addition, the present invention relates to a process for preparing recombinant proteins by using yeast expression vector with XbaI cleavage site inserted in order to facilitate to insert the

recombinant protein genes.

By using the present expression vector, the recombinant proteins such as human granulocyte colony-stimulating factor(hGCSF) and human growth hormone(hGH) can be prouced with high secretion efficiency. An experiment of bone marrow differentiation and proliferation has disclosed that the colony of neutrophilic granulocyte or monocytic macrophage is formed, and thereafter it has been known that colony stimulation factors exist in the living body [J. Cell. Comp. Physiol. 66: 319 (1965); Aust. J. Exp. Biol. Med. Sci. 44: 287 (1966)].

The factors called as colony stimulating factor (hereinafter it refers to "CSF")are classified by their characteristics in biological activity as follows:

(i) GM-CSF (granulocyte-macrophage CSF) proliferates and differentiates stem cell of granulocytic leucocyte and monocytic macrophage, and finally forms colonies, (ii) M-CSF (macrophage CSF) forms colony of monocytic macrophages, iii) multi-CSF (multi-lineage CSF) stimulates undifferentiated pluripotent stem cells and finally forms colony of pluripotent cells, (iv) G-CSF (granulocyte CSF) forms colony of granulocyte

leucocytes [J. B. C. 252 : 1998-2033 (1977), J. B. C. 252: 4045-4052 (1977), Biochem. J. 185 : 341-343 (1980), J. B. C. 258 : 9017-9021 (1983)].

GCSF, about 20kDa glycoprotein, is derived from monocyte, monocytic macrophage, epithelial cell, fibroblast etc. And human GCSF (hereinafter it refers to "hGCSF") gene exists on 17th chromosome. It is known that GCSF stimulates production of neutrophilia colony in vitro and of colonies of blast cell, macrophage in cooperation with IL-3, and get some myeloid leukemic cell line matured. GCSF increases the number of neutrophil and monocyte in vitro.

The clinical applications of hGCSF are as follows :

First, hGCSF increases the number of neutrophil dosage-dependently in treating a neutropenic patients with advanced solid and hematologic malignances.

Second, hGCSF recovers patients rapidly from neutropenia by chemotherapy for malign lymphatic tumor, lung cancer, testis cancer, urethra epithelioma and acute leukemia etc.

Third, hGCSF increases the number of neutrophil upon bone marrow transplantation for acute nonlymphocytic leukemia and chronic bone marrow

leukemia patient.

Fourth, hGCSF recovers patients rapidly from neutropenia due to bone marrow dysplasia syndrome.

Fifth, hGCSF recovers patients rapidly from neutropenia due to aplastic anemia.

Sixth, hGCSF is useful for hereditary and idiopathic neutropenia.

Seventh, hGCSF prevents or reduces the incidence of mucositis and febrile neutropenia due to anti-tumor chemical treatment (Drug Evaluations Annual 1993, American Medical Associations p2232-2333).

Human growth hormone (hereinafter it refers to "hGH") is nonglycosylated protein which is made up of 191 amino acids, and it is secreted from pituitary anterior lobe. The hGH containing 2 intramolecular disulfide bonds has 22,000 dalton of molecular weight. It is initially synthesized as a precursor and is secreted from the cell after processing.
The hGH is produced in large quantities before adulthood, and is produced during a whole human life.

The hGH is necessary for normal growth and development, but several types of dwarfism are caused by the abnormal low-level production of hGH and the

over production of hGH can be accompanied by acromegaly or gigantism.

The hGH shows various biological activities and reacts to various tissues, directly or indirectly. It has an effect on linear bone growth rate and lactation, and shows diabetogenic insulin-like activity. In addition, it promotes protein synthesis, and has an effect on metabolism of lipid and carbohydrate.

The followings are clinical applications of hGH :

It is known that abnormal growth can be recovered if the hGH is administrated at the childhood in the case of dwarfism caused by deficiency of hGH [Raben, M. S., J. Clin. Endocr. 18 : 901-904 (1958)]. It is known that hGH is also used for treatment of obesity, and effective on treatment of various ailments such as bone fracture, skin burn, bleeding ulcer etc [Proc. of NIAMDD Symp. Publ. No. 74-612 (ed. Raiti, S.) (Baltimore, Maryland, 1973)].

Base sequence of hGH DNA is known by cDNA cloning of this gene, and the expression of hGH DNA in *E. coli* has been reported [Martial et al., Science 205 : 602-605 (1979)].

Many genetic engineering methods have been

attempted for the overproduction of recombinant proteins.

First, a method of expressing protein in *E. coli* after cloning the target gene has been developed [Science 232: 61-64 (1986)]. But there are some disadvantages in the method using *E. coli* as a host as described in the followings.

In a human body, protein is synthesized as precursor first and then is processed to mature form by proteolysis.

But when the protein is expressed in *E. coli*, the N-terminal methionine of the synthesized protein is not so effectively removed by the aminopeptidase as in the human body and hence the proteins with and without the methionine can coexist in the cytoplasm of *E. coli*. Then it is very difficult to separate the protein without methionine from the protein with methionine.

In many cases, protein is expressed in inactive, or insoluble form and then it should be converted to biologically active protein through a renaturation(refolding) process where the recovery yield of protein is sometimes significantly reduced.

And there is a problem of contamination by bacterial endotoxin in the purification process.

In addition, the post-translational modification of protein (e.g. glycosylation of hGCSF) is not possible in *E. coli*.

Secondly, the cloned target gene has been expressed in animal cell such as CHU-2 (human GCSF-producing tumor cell line) or Chinese hamster ovary cell.

But the method using animal cell as a host has such disadvantages that culture condition is complicated with expensive serum media and recovery yield is generally very low since small amount of recombinant protein is usually purified from large volume of culture media [EMBO J. 5: 871-876 (1980)], (KR 91-5624).

As a plausible solution to the above problems, the expression system using yeast as a host has been developed. The method that can obtain target polypeptides or proteins in large amounts from recombinant yeast has been reported by Loison and others [Bio/Technol. 4 :433-437 (1986); Burrow, "Baker's yeast, p349-420, in The Yeast, vol. 3, Rose and Harrison, eds. Academic Oress, London (1970)]. The expression system of recombinant yeast has

significant advantages compared to the other expression systems employing animal cell or *E. coli* as a host.

The present inventors have studied a process of preparing hGCSF by using yeast. U. S. FDA noticed that yeast is not pathogenie to human body and most of regulation principles of gene expression in yeast are disclosed [Strathern et al., The Molecular Biology of the Yeast Saccharomyces, Metabolism and Gene Expression, Cold Spring Harbor Laboratory, N.Y. (1982)].

Using yeast as host cell has advantages that it is generally regarded as safe organism to human body, and that it is possible to produce the large amount of hGCSF from high cell density cultures, and the purification process is simplified because soluble protein is secreted from the cells, being directed by the signal peptide.

Recently methods of expressing heterologous proteins such as B-type hepatitis virus, inteferon, calf chymosin, epidemal growth factor in yeast have been reported [Valensuela et al., Nature 298: 347-350 (1982); Hitzeman et al., NAR 11: 2745-2763 (1983); McAleer et al., Nature 307: 178-180(1984) ; Tuite et

al., EMBO, J. 1: 603-608 (1982); Mellor et al., Gene 24: 1-14 (1983); Urdea et al., PNAS 80: 7461-7465 (1983)].

But the expression level of heterologous proteins in recombinant yeast is generally low very in comparison with that of homologous proteins in yeast, and therefore, the extensive efforts for developing the efficient expression vectors have been make to increase the expression level of heterologous in proteins in yeast [Chen et al., NAR 12: 8951-8970 (1984)].

For example, EP 84303833 discloses a process to prepare galactokinase-bovine prochymosin fusion protein from yeast by using a cloning vector with foreign target gene and yeast GAL1 promoter. Also in case that GAL4 gene of yeast is inserted to the expression vector containing exogenous gene and GAL1 promotor, the expression of GAL4 protein is increased via the transcription-level control by galactose, and hence the synthesis of the foreign protein can be increased [Laughon et al., PNAS 79: 6827-6831 (1982)].

EP 84302723 discloses a method of expressing human interferon, human serum albumin, bovine interferon α-1, α-2, tissue plasminogen activator, rennin, and human insulin-like growth factor in yeast

by using the signal sequence and promoter of mating factor α.

Summary of the Invention

The object of the present invention is to provide a process for preparing recombinant proteins from yeast by using recombinant DNA technology. Precisely, the present invention provides to a process for preparing recombinant proteins by using yeast expression vectors which consist of hybrid promoter consisting of two kinds of yeast inducible promoters and secretory signal consisting of yeast killer toxin and amino terminal of mature interleukin 1ß(IL-1ß).

The object of the present invention is to provide a process for preparing hGCSF from yeast by using expression vector made up of promoter and secretory signal of yeast heat shock protein 150.

The object of the present invention is to provide a process for preparing recombinant proteins by using yeast expression vector with XbaI cleavage site inserted in order to facilitate to insert the recombinant protein genes.

Brief description of drawing

In the accompanying figures ;

Fig. 1 shows a process for preparing YEp2-k

Fig. 2 shows a process for preparing YEp2KIL20GC

Fig. 3 shows a process for preparing pIL20GC

Fig. 4 shows a process for preparing YEpHSPGC

Fig. 5 shows an amino acid sequence consisting of killer toxin leader, N-terminal 24 residues of IL-1ß, and hGCSF, and cleavage sites digested by signal peptidase and by KEX2 peptidase

Fig. 6 shows a SDS-PAGE analysis of hGCSF expressed in yeast

Fig. 7 shows a result of western blotting of hGCSF which is expressed in yeast by using yeast expression vector, YEpHSPGC

Fig. 8 is a gragh showing the time-course variation in cell, ethanol, and hGCSF concertrations and plasmid stability.

Fig. 9 shows a SDS-PAGE analysis of hGCSF expressed in yeast

Fig. 10 is a gragh showing the time-course variation in cell, ethanol, and hGH concentrations and plasmid stability.

Fig. 11 shows a SDS-PAGE analysis of hGH expressed in yeast

Fig. 12 shows an amino acid sequence consisting of killer toxin leader, N-terminal 24 residues of IL-1 , and hGH, and cleavage sites digested by signal peptidase and by KEX2 peptidase

Fig. 13 is a map of pIL20XGH

Fig. 14 shows a purification process of hGCSF in yeast culture broth

Fig. 15 shows a purification process of hGCSF via Sephacryl S-200 column chromatography

Fig. 16 shows the last step in the purification process of hGCSF

Detailed Description of the Invention

The present inventors pay attention to the fact that for the high-level production of recombinant hGCSF from yeast, the secretion efficiency should be enhanced as well as the expression level.

In order to secret a processed hGCSF, various secretory signals were fused to amino-terminal of hGCSF but the secretion was not successful.

Meanwhile it has been reported that interleukin 1ß is efficiently secreted from yeast with

a secretory signal [EMBO J. 6: 229-234 (1987)]. The present inventors paid attention to the possibility that amino acids of IL-1ß amino terminal may be useful for secreting the processed hGCSF out of the yeast cell. The present inventors have finally found that hGCSF is successfully expressed and secreted out of cell when the fusion peptide consisting of a killer toxin secretory signal and 24 amino acids of IL-1ß is placed in front of hGCSF gene. At this time, the dibasic KEX2 cleavage site was inserted between the N-terminal 24 residues of IL-1ß and hGCSF and thereby, the mature hGCSF was released by proteolytic action of KEX2 enzyme (endopeptidase) in the secretory pathway.

The expression vector described above has arrangement of killer toxin secretory signal-24 amino acids of IL-1ß-KEX2 cleavage site mature hGCSF. The protein expressed by using the above expressing vector is secreted through the following steps: the signal peptide is digested by signal peptidase during the translocation of the synthesized protein into Golgi, IL-1ß region is excised by KEX2 peptidase, and then mature hGCSF with correct amino-terminal sequence is finally secreted.

The hGCSF expression vector used in the present

invention will be described in detail as follows.

The hGCSF expression vector comprises: mating factor-α1 promoter replacing CYC-1 promoter in yeast expression vector YEpsec1-hI1 [C. Baldari et al., EMBO J. 6: 229-234 (1987)], hybrid secretory signal consisting of killer toxin leader sequence which is optimized by yeast codon usage and 24 amino-terminal of IL-1ß, hGCSF gene, and GAL4 which is GAL gene activator. Saccharomyces cerevisiae is transformed by this expression vector and then selected transformant in uracil-defective minimal media is used as a hGCSF-producing strain. As a result of recombinant gene expression in high cell dinsity cultures of this selected transformant, the extracellular hGCSF was produced in large amount, and the culture conditions for the cell growth and the hGCSF production in fermenter were systematically optimized.

The present inventors also have developed an expression vector consisting of promoter and signal leader peptide of heat shock protein, with which the expression of recombinant hGCSF is regulated by temperature-shift. Differently from other inducible yeast promoters such as GAL promoter induced by galactose, Pho 5 promoter induced by

phosphorus-starvation, ADHII promoter induced by glucose-starvation, the promoter of heat shock protein (HSP) regulates the transcription and hence protein synthesis only by temperature control (37~42°C). And the HSP is secreted from the cells by leader sequence (PNAS, 89 : 3671-3675). The present inventors have developed a method of preparing hGCSF by using expression vector constructed by HSP150 promoter and leader sequence of HSP.

In addition, the inventors inserted XbaI site between the amino acid sequence of IL-1ß and the KEX2 cleavage site in the mating factor α promoter-based expression vector described above for the purpose of facilitating the insertion of other heterologous genes.

The above expression vector can be used for the production of other recombinant proteins. Particulary in this invention, the structural gene of hGH was inserted into the expression vector above by using XbaI-BamHI fragment as a cloning site and the hGH was successfully expressed from the selected transformant. Also, in the high cell ensity cultures of the selected transformant above, the hGH was successfully synthesized and secreted into the extracellular broth of fermenter in large quantity.

Ii is important to mention here that other

recombinant proteins can be produced using the expression vector above although in this invention, the methods for producing hGCSF and hGH are only presented.

The amount of hGH production in the above high cell density fermentation is more than 1g/L, which is relatively very high level compared to the fermentation yield of other yeast-derived recombinant proteins, reported in the past. Therefore, it may be possible that other recombinant proteins are expressed an efficiently secreted from yeast by employing the expression vector using the sequence comprising killer toxin leader sequence-amino terminal 24 residues of IL-1ß-hGH as a hybrid signal peptide.

The present invention will be described in detail with examples.

Examples are only for showing this invention, but does not limit the range of the claims of the present invention.

1. The preparation of YEp2-k.

Expression vector YEpsecl-hI1 consists of upstream activation sequence of GAL1, 10 gene, CYC-1 promoter, killer toxin leader sequence of Kluyveromyces

lactics [M. J. R. Stark et al., NAR 12 : 6011-6031 (1984)] and interleukin-1ß gene, and IL-1ß is expressed by the inducer, galactose.

To make vector YEpsec1-hI1 more effective, killer toxin leader sequence was optimized, and CYC-1 promoter was substituted with more effective MFα1 promoter.

In order to terminate mRNA transcription, transcription terminator of GAPDH is added to the 3' terminal of hGCSF, and Gal 4 gene which is activator of Gal gene, is cloned and added into the expression vector.

1) Optimization of killer toxin leader sequence codon

The formation of YEpsec-ok

<Example 1> Synthesis of oligonucleotide of killer toxin leader sequence.

In order to substitute codons of killer toxin leader sequence of yeast expression vector YEpsec1-hI1 with the codons which encode proteins which are overexpressed in *Saccharomyces cerevisiae*, the following oligonucleotide was synthesized by synthesizer (ABI, 392 DNA/RNA synthesizer) (J. Bennetzen, B. Hall J. Biol. Chem. 257 : 3026-3031).

C TAT AAA ACA ATG AAC ATC TTC TAC ATC TTC TTG

TC GAG ATA TTT TGT TAC TTG TAG AAG ATG TAG AAG AAC

SacI

TTC TTG TTG TCT TTC GTT CAA GGT AC

AAG AAC AAC AGA AAG CAA GTT C

KpnI

To insert the synthesized oligonucleotide into the site at which killer toxin leader sequence of YEpsec1-hI1 is cut out, the following reactions are followed. Each 5' terminus of oligonucleotide was phophorylated by T4 polynucleotide kinase(NEB) in the 30μl of reaction solution [70mM Tris-HCl(pH 7.6), 10mM MgCl2, 5mM DTT(dithiothreitol)] containing ATP at 37°C for 1hr. Two reaction solutions were mixed and were left for 20 minutes. The oligonucleotides were annealed, while cooling to 30°C slowly.

<Example 2> The digestion of YEpsec1-hI1

1μg of YEpsec1-hI1 was digested with restriction enzymes(SacI, KpnI; NEB) at 37°C for 1hr in the 40μl reaction solution (20mM Tris-acetate, 10mM magnesium acetate, 50mM potassium acetate), and then separated by electrophoresis in 1% slab agarose gel. After separation, 8.4kb band was sliced and was eluted

from the sliced DNA band by using Jetsorb(GENOMED, cat # 110300), and was purified.

<Example 3> The ligation of DNA and transformation

The oligonucleotides of killer toxin leader sequence annealed in the example 1 and YEpsec1-hI1 digested with restriction enzymes, SacI and KpnI in the example 2 were ligated by 100 unit of T_4 DNA ligase in 30μl reaction solution, consisting of 50mM Tris-HCl, 10mM MgCl2, 10mM DTT, and 1mM ATP at 16°C overnight. *E.coli* XL-1 Blue (supE44 hsdR17 recA1 end A1 gyrA46 thi relA1 Iac$^-$F'[proAB$^+$ lacI^q lacZΔ M15 Tn(tetr)]) was transformed with ligation reaction solution by CaCl2 method according to Molecular Cloning A Laboratory Manual(Sambrook, Fritch Mantiatis, 2nd adition, CSH). After transformation, transformed *E.coli* was spread on the LB-Amp agar plate media(10g/1 trypton, 5g/1 yeast extracts, 10g/1 NaCl, 100μg/ml ampicilin), andincubated for 20hrs at 37°C. After the colony of ampicilin-resistant transformant(Amp^R) was cultured in the 1.5ml of liquid LB-Amp media, the plasmid was eluted by alkali lysis method and purified by using RPM rotation filter (BIO 101). The plasmid which is not digested by restriction enzyme SmaI is selected and named as YEpsec-ok because the restriction enzyme SmaI

site disappears on the killer toxin leader sequence by the step of the codon optimization.

<Example 4> Single stranded DNA

To identify the base sequence of killer toxin leader sequence substituted for the purpose of codon optimization, the sequenscing of YEpsec-ok was conducted. The single stranded DNA necessary for sequencing is prepared as follows.

YEpsec-ok obtained in the example 3 was digested by restriction enzyme BamH1, SacI again, and electrophoresed at the 1.5% agarose gel, and 0.66kb DNA fragment was sliced. After this DNA was eluted from agarose gel using GENE CLEAN KIT II. 1μg of vector M13mp19 was digested by restriction enzyme BamHI, SacI, and then was purified by using GENE CLEAN KIT II. 0.66kb DNA and M13mp19 was ligated by T_4 DNA ligase in the ligation mixture for 16°C overnight. After competent *E.coli* XL-1 Blue was transformed with a reaction solution, single-stranded DNA was isolated according to the Molecular Cloning A Laboratory Manual(ibid). More specifically, 200μl of XL-1 Blue solution cultured overnight, 40μl of X-Gal (20mg/ml in dimethylformamide) and 4μl of IPTG (200mg/ml) were mixed with agar, the mixture was spread on LB agar

plate media. After the transformed *E.coli* was incubated at 37°C, one white plaque was picked on the agar plate. The picked plaque infected 200μl XL-1 Blue with 20ml LB at 37°C, and cultured for 5hrs at 250rpm. After this, the cultured solution was centrifuged, and 1/5 volume of PEG (20% PEG 8000 in 2.5M Nacl) was added to supernatant and left on ice for 15minutes. After centrifugation the supernatant was discarded. Precipitated M13 virus pellet was suspended in 200μl of TE buffer solution(10mM Tris-HCl(pH 7.6), 1mM EDTA) and then protein was extracted with phenol/chloroform/isoamylalcohol(25:24:1). After centrifugation, 2 volume of ethanol was added to supernatant to precipitate DNA, and DNA pellet was washed with 70% ethanol. After the pellet was dried by vacuum, the pellet was dissolved in 20μl of distilled water.

<Example 5> Sequence analysis

The base sequence of single-stranded plasmid prepared in the example 4 was analyzed by dideoxy chain termination DNA sequencing method. The primer used in sequeucing was synthesized with ABI synthesizer.

Oligo DNA for analysis of base sequence

5′ GTT TTC CCA GTC ACT AC 3′

According to the result of analysis of base sequence, it is shown that the bases of the codons were substituted into the optimized codon.

GAG CTC TAT AAA ACA ATG AAC ATC TTC TAC ATC TTC
SacI
TTG TTC TTG TTG TCT TTC GTT CAA GGT ACC CGG GGA
KpnI
TCA CTG AAC

2) The preparation of transcription terminator of GAPDH(glyceraldehyde 3-phosphatase dehydrogenase) (YEpsec-term)

<Example 6> Synthesis of oligonucleotide

In order to terminate the transcription by GAL1, 10 UAS(upstream activation sequence)-MFα1 promoter, the transcription terminator of GAPDH was synthesized as the following[J. Biol. Chem. 245:839-845(1979)].

GA TCC CGG GTT TTT TAT AGC TTT ATG ACT TAG TTT CAA
G GCC CAA AAA ATA TCG AAA TAC TGA ATC AAA GTT
BamHI
TTA TAT ACT ATT TTA ATG ACA TTT TCA GG
AAT ATA TGA TAA AAT TAC TGT AAA AGT CCA GCT
SalI

After synthesis, the oligonucleotides were purified by OPC(oligo purification column), and were phosphorylated and annealed according to the example 1.

<Example 7> Digestion of YEpsec1-hI1

In order to insert the transcription terminator of GAPDH to the downstream of expressed gene, YEpsec1-hI1 was digested by restriction enzyme BamHI, SalI at 37°C for 1hr. The digested plasmid was electrophoresed in 1% slab agarose gel, and 9kb band was sliced, and DNA was eluted from the cut band by using Jetsorb.

<Example 8> Ligation of DNA and transformation

YEpsec1-hI1 digested by restriction enzyme BamH1, SalI and transcription terminator oligonucleotide of GAPDH which is phosphorylated and annealed in the example 6 were ligated by T_4 DNA ligase at 16°C.

Ampicilin-resistant transformed colony obtained by transforming *E.coli* XL-1 Blue by reaction solution according to $CaCl_2$ method was cultured in the 1.5ml LB-amp media. After culture, the plasmid was purified by RPM filter.

Plasmid was digested by restriction enzyme BamH1, Sal1, and was electrophoresed in 8% PAGE(Polyacrylamide gel electrophoresis). The plasmid with 70bp DNA band was named as YEpsec-term.

3) Preparation of YEpsec-ok

<Example 9> Digestion of YEpsec-ok

1μg of YEpsec-ok was digested by restriction enzyme KpnI, SalI at 37°C for 1hr, and was separated in 1% slab agarose gel. About 8.3kb band was sliced and DNA was purified by Jetsorb.

<Example 10> Digestion of YEpsec-term

1μg of YEpsec-term is digested by restriction enzyme KpnI, SalI at 37°C for 1hr. After this, plasmid was separated in 1% slab agarose gel and the 0.6kb band was sliced and DNA was eluted.

<Example 11> Ligation of DNA and transformation

0.6kb of fragment eluted in the example 10 and 8.3kb of vector eluted in the example 9 were dissolved in 30μl of ligation solution, and was ligated by T_4 DNA ligase. The *E.coli* XL-1 Blue was transformed with reaction solution by $CaCl_2$ method. Ampicilin-resistant transformed colony was cultured in the 1.5ml of LB-Amp media, and plasmid was isolated by RPM rotation filter. The plasmid was digested by restriction enzyme, and the plasmid with transcription terminator to the YEpsec-ok was named as YEpsec-kt.

4) Substitution CYC-1 promoter with MFα1 promoter (The preparation of Yep αkt)

The original YEpsec1-hI1 consists of complex promoter of CYC-1 promoter and regulation region, GAL1-10 UAS on which Gal4 protein, activator of genes related to galactose metabolism, binds, but in this invention the CYC-1 promoter was substituted with MFα1 promoter which has more effective transcription initiation [Kurjan et al., Cell. 30 : 933-943 (1982)].

<Example 12> PCR(polymerase chain reaction) of MFα1 promoter

For the convenience of cloning, MFα1 promoter

was obtained by PCR, using primers which has proper restriction enzyme site.

At the 5' terminus of each primer, there are restriction enzyme SalI and SacI cutting site.

Primers for amplification of transcription initiation sequence of MFα1.

5′ GTG CAC TCG AGC CAA AAA GCA ACA ACA GGT TTT GG 3′
SalI

5′ TTA ATG AGC TCT ATT GTG TAT GAA ATT GAT AGT TTG3′
SacI

The template used in PCR was p70αT vector containing MFα1 promoter. 2 units of Vent DNA polymerase was added to 50 pmol of each primer and 100μl of reaction solution [10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl(pH 8.8), 2mM $MgSO_4$, 0.1% triton X-100] including 200μM dNTP, and then the reaction was cycled 35 times by using PCR ROBOT(Fine Co.), with the following temperature programme :

Pretreatment 94°C , 300 seconds ;

Annealing 53°C, 40 seconds ;

Extension 72°C, 40 seconds ;

Denaturation 94°C, 40 seconds ;

Postreaction 53°C, 300 seconds

Amplified MFα1 promoter was electrophoresed in 1.5% slab agarose gel, and 150bp DNA band was identified and eluted from the gel. Eluted DNA was digested with restriction enzyme SalI and SacI.

<Example 13> Digestion of YEpsec-kt

1μg of YEpsec-kt was digested with restriction enzyme, XhoI and SacI at 37°C for 1hr. Digested plasmid was separated in 1% slab agarose gel and about 8.8kb DNA band was sliced and DNA was eluted using Jetsorb.

<Example 14> Ligation and transformation

MF α1 promoter which was prepared by digesting with SalI and SacI in the example 12 and 8.3kb of vector which was digested with XhoI and SacI in the example 13 was ligated by T_4 DNA ligase in 30μl of ligation solution. *E.coli* XL-1 Blue was transformed by reaction solution, and ampicilin-resistant transformed colony was cultured in 1.5ml LB-Amp media, and plasmid was purified. Because plasmid can not be cut by XhoI and SalI when the XhoI site and SalI site is ligated correctly, the plasmid which can not be cut by XhoI, SalI was selected and named as YEpαkt.

5) Gal4 gene (The preparation of YEpαktGAL4)

When galactose is carbon source, genes related to galactose metabolism (Gal7,10,1, Gal2, Mel1) is expressed by activator GAL4 protein in yeasy [Johnstone et al., Proc. Natl. Acad. Sci. USA. 79 : 6971-6975 (1982)].

Such induction begins at the transcription level of each gene, and GAL4 protein acts as transcription activator. Gal1(kinase)-Gal10(epimerase) site of YEpsec1-hI1 contains UAS which is binding site of GAL4 [Cirton et al., J. Bacteriol. 158 : 269-278 (1984)]. YEpsec1-hI1 is yeast 2μ circle high-copy number plasmid. Because GAL4 protein is encoded by chromosomal DNA, the concentration of GAL4 protein which can bind at GAL1-10 USA is low when induced by galactose. It is difficult to express sufficiently. In order to maintain sufficient amount of GAL4 protein, GAL4 gene was inserted into YEpsec1-hI1.

<Example 15> PCR of GAL4 gene

For PCR of GAL4 gene the following primers were synthesized.

5'AGTTTGAATTCCAACAGCAAGCAGGTGTGCAAGACA3'
EcoRI

5'TCGAAGAATTCTCACCTTCGTGAACTTCAGAGGCGA3'
EcoRI

The template was genome DNA of *Saccharomyces cerevisiae* 2805.

The PCR has the following composition : 50 pmol of each primer, 100μl of reaction solution[10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl(pH 8.8), 6mM $MgSO_4$, 0.1% triton X-100] including 200μM dNTP, and the used polymerase was 2 units of Vent DNA polymerase by using PCR ROBOT (Fine Co.). The reaction is cycled 35 times, with the following temperature programme :

Pretreatment 94°C, 300 seconds ;

Annealing 53°C, 50 seconds ;

Extension 72°C, 260 seconds ;

Denaturation 94°C, 50 seconds ;

Postreaction 53°C, 300 seconds

Amplified GAL4 gene was electrophoresed in 1% slab agarose gel, and about 3.5kb DNA band was identified, and DNA was eluted from gel and digested by restriction enzyme EcoRI.

<Example 16> Preparation of pUCGAL4

After pUC18 was digested by restriction enzyme, EcoRI at 37°C for 1hr, digested plasmid was dephosphorylated by CIP (calf intestinal phosphatase, NEB). The plasmid digested by EcoRI was purified by

using Jetsorb. The GAL4 gene and pUC 18 digested by EcoRI was ligated by T_4 DNA ligase in 30μl of ligation solution at 16°C overnight. *E.coli* XL-1 Blue was transformed according to $CaCl_2$ method, and colony was cultured, and plasmid was purified by RPM rotation filter. When the plasmid was digested by EcoRI, the one with 3.5kb DNA band was selected and named as pUCGAL4.

<Example 17> YEpαktGAL4

In order to insert GAL4 gene into YEpαkt, the following procedures were conducted.

pUCGAL4 was digested by restriction enzyme, EcoRI, and electrophoresed in 1% slab agarose gel, and about 3.5kb DNA band was sliced, eluted from gel and purified. 1μg of YEpαkt was digested by restriction enzyme, EcoRI at 37°C for 1hr, and the plasmid was dephosphorylated by CIP(calf intestinal phosphtase, NEB), and digested vector was purified by using Jetsorb. The GAL4 gene and YEpαkt digested by EcoRI was ligated by T_4 DNA ligase in the ligation solution at 16°C, overnight. *E.coli* XL-1 Blue was transformed according to $CaCl_2$ method, and colony was cultured and then plasmid was purified. When the plasmid was cut by EcoRI, 3.5kb band was selected and named as YEpαktGAL4.

6) Preparation of YEp2-k

<Example 18> Preparation of YEp2-k

There are two digestion-site of restriction enzyme, KpnI on YEpαktGAL4. One is on the terminus of killer toxin leader sequence, and the other is on the selection marker, leu2-d gene. In this invention, since URA3 is used as selection marker of yeast, digestion-site of KpnI which is on leu2-d gene was destroyed. After YEpαktGAL was digested with KpnI partially, the digestion sites were filled by T_4 DNA polymerase in 50μl of reaction solution[10mM Tris-HCl pH 8.0, 5mM MgCl2, 5mM DTT, 100μM dNTP, 50μg/ml BSA] at room temperature for 1hr, and the KpnI site became blunt-ended. The site of blunt-ended KpnI was ligated by T_4 DNA ligase and ATP at 16°C overnight. *E.coli* XL-1 Blue was transformed according to $CaCl_2$ method, and colony was cultured in 1.5ml LB-Amp media, and then plasmid was purified. Plasmid was digested with restriction enzyme, and the plasmid which has not KpnI site on leu2-d gene was named as YEp2-k.

II. Cloning of GCSF (Preparation of YEp3KGC)

<Example 19> Preparation of hGCSF

For the PCR of GCSF, the oligonucleotide was

synthesized as follows.

5′ AGG TAG GGT ACC ACC CCC CTG GGC CCT GCC 3′
KpnI

5′ ATG GGA GGA TCC GGG CTT GGC TCA GGG CTG GGC 3′
BamHI

Template used for PCR of hGCSF is macrophage cDNA library (Clontech). 2 units of Vent DNA polymerase was added to 50pmol of each primer and 100μl of reaction solution [10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl (pH 8.8), 2mM $MgSO_4$, 0.1% Triton X-100] including 200μ dNTP and then the reaction was cycled 35 times by using PCR ROBOT (Fine Co.) with the following temperature programme :

Pretratment 94°C, 300 seconds ;
Annealing 53°C, 30 seconds ;
Denaturation 94°C, 30 seconds ;
Postreaction 53°C, 300 seconds

Amplified hGCSF gene was electrophoresed in 1 % slab agarose gel, and 0.5 kb of DNA band was identified, purifed and digested with KpnI and BamHI.

<Example 20> Digestion of YEpsec-kt

After 1μg of YEp2-k was digested with KpnI and

BamHI, the digested DNA was electrophoresed in 1 % slab agarose gel, and fragment of IL-1ß was discarded and the rest of fragment of vector was eluted.

< Example 21 > DNA ligation and transformation

GCSF gene and YEp2-k digested with KpnI and BamHI was ligated by T_4 DNA ligase in 30μl of ligation solution. *E.coli* XL-1 Blue was transformed by reaction solution, and colony was cultured and then plasmid was purified. When the plasmid was digested with restriction enzyme, KpnI and BamHI, the plasmid which inserted fragment of GCSF was named as YEp2KGC.

III. Preparation of YEpGalMF

In order to express GCSF in general cloning vector, YEp352 [J. E. Hill et al., Yeast 2: 163-167 (1986)] the following procedure is conducted. The fragment necessary for expression of GCSF was obtained from YpGX265Gal4, and expression vector, YEpGalMF, was formed and used in expression of GCSF.

< Example 22 > PCR of GAL4-GAL1, 10 UAS-MF 1

UAS-MFα1 in the GAL4-GAL1, 10 was obtained from the YpGX265GAL4 [US patents No. 5,013,652] by PCR, using the primer complementary to GAL4 and promoter of

MFα1.

primer complementary to promoter of MFα1

5′ TTAATGAGCTCTATTGTGTATGAAATTGATAGTTTG3′

SacI

primer complementary to GAL4.

5′AGTTTGAATTCCAACAGCAAGCAGGTGTGCAAGACA3′

EcoRI

2 units of Vent DNA polymerase was added to 500 pmol of each primer, 100μl of reaction solution including 200μl of dNTP, and then the reaction was cycled 35 times by using PCR ROBOT(Fine Co.) with the following temperature programme:

Pretratment 94°C, 300 seconds;

Annealing 53°C, 30 seconds;

Extension 72°C, 30 seconds;

Denaturation 94°C, 30 seconds;

Postreaction 53°C, 300 seconds

Amplified GAL4-GAL1, 10 UAS-MF 1 gene was electrophoresed in 1 % slab agarose gel, about 4 kb of DNA band was identified, eluted from gel and digested with SacI and EcoRI.

<Example 23> Ligation of YEp352

1 μg of YEp352 was digested by SacI and EcoRI at 37°C for 1 hr, and plasmid was electrophoresed in 1 % slab agarose gel, and about kb DNA band was sliced and eluted by using Jetsorb.

Pretreatment 94°C, 300 seconds;

Annealing 50°C, 30 seconds

Extension 72°C, 30 seconds;

Denaturation 94°C, 30 seconds

Postreaction 53°C, 300 seconds

<Example 24> DNA ligation and transformation

The GAL4-GAL1, 10 UAS-MFα1 of YpGX256GAL4 and YEp352 digested with SacI, EcoRI was ligated by T_4 DNA ligase in the 30μl of ligation solution. *E.coli* XL-1 blue was transformed with reaction solution, and colony was cultured and then plasmid was purified. When the plasmid was digested with restriction enzyme, SacI, EcoRI, the 3.5 kb band was selected and named as YEpGalMF.

IV. Preparing of YEp2kIL20GC

< Example 25 > Preparing of IL20GCSF

1) The following primer was synthesized for PCR of killer toxin leader sequence, 24AA of amino

terminus of IL-1ß and cleavage site of endopeptidase KEX2.

5′ ACA ATA GAG CTC TAT AAA ACA 3′

SacI

5′ GGC AGG GCC CAG GGG GGT TCT CTT GTC CAA AGA AAC AGC TTT CAG TTC ATA TGG 3′

YEp2-k was used as template. 2 units vent DNA polymerase was added to 50 pmol of each primer, 100μl of PCR reaction solution including 200μM of dNTP and then the reaction was cycled 35 times, with the following programme:

Pretreatment 94°C, 300 seconds;

Annealing 50 °C, 30 secons;

Extention 72°C, 30 seconds;

Denaturation 94°C , 30 seconds;

Postreaction 53°C, 300 seconds

Amplified killer toxin leader sequence, IL-ß24 AA, was electrophoresed in 1.5 % slab agarose gel, and about 80 bp DNA band was sliced, eluted from gel and purified. The DNA sequence amplified by PCR is as follows.

ACA ATA GAG CTC TAT AAA ACA MET ACC TTC TAC ATC

TGT TAT CTC GAG ATA TTT TGT TAC TGG TAG AAG ATG TAG

SacI

TTC TTG TTC TTG TTG TCT TTC GTT CAA GGT TTG TCA CTG
AAG AAC AAG AAC AAC AGA AAG CAA GTT CCA AAC AGT GAC

AAC TGC ACG CTC CGG GAC TCA CAG CCA AAA AGC TTG GTG
TTG ACG TGC GAG GCC CTG AGT GTC GGT TTT TCG AAC CAC

ATG TCT GGT CCA TAT GGA CTG AAA GCT GGT GTT TCT TTG
TAC AGA CCA GGT ATA CCT GAC TTT CGA CCA CAA AGA AAC

GAC AAG AGA ACC CCC CTG GGC CCT GCC 3′
CTG TTC TCT TGG GGG GAC CCG CGA CGG

2) GCSF is amplified by PCR by using the product of PCR described above 1) and oligonucleotide complementary to carboxy terminus of GCSF.

The oligonucleotide complementary to carboxy terminus of GCSF was synthesized as follows.

5′ATG GGA GGA TCC GGG CTT GGC TCA GGG CTG GGC 3′

BamHI

YEp2KGC obtained from example 21 was used as template. 2 units Vent DNA polymerase was added to 50

pmol of each primer, DNA, of killer toxin sequence, 24AA of IL-1ß and 100μl of reaction solution including 20μM of dNTP, and then the reaction was cycled 35 times, with the following temperature programme:

Pretreatment 94°C, 300 seconds;

Annealing 50 °C, 30 seconds

Extension 72°C, 30 seconds:

Denaturation 94°C, 30 seconds;

Postreaction 53°C, 300 seconds

Amplified IL20GCSF was electrophoresed in 1 % slab agarose gel, and about 0.66 kb DNA band was sliced, eluted from gel and purified. IL20GCSF was digested with SacI, BamHI and purified.

< Example 26 > EDigestion of YEp2-k

1μg of YEp2-K was digested with SacI and BamHI at 37°C for 1hr. Digested plasmid was electrophoresed in 1% agarose gel, and about 12 kb DNA band was sliced and DNA was eluted by using Jetsorb.

< Example 27 > DNA ligation and transformation

The IL20GCSF and YEp2-k digested with SacI and BamHI in the example 26 were ligated by T_4 DNA ligase in 30μl of ligation solution.

E. coli XL-1 Blue was transformed by the reacted solution, then colony was cultured, and the plasmids were purified. Plasmids were digested with restriction enzyme, and only the plsmids containing IL20GCSF was selected and named as YEp2kIL20GC.

V. Preparation of pIL20GC

< Example 28 > Digestion of YEp2kIL20GC

To obtain IL20GCSF-GAPDH transcription terminator from YEp2kIL20GC, the plasmid was digested with KpnI and SalI at 37°C for 1 hr. The digested plasmid was separated by the same method as above, and DNA with the size of about 0.66kb was purified.

< Example 29 > Digestion of YEpGalMF

1 μg of YEpGalMF was digested by KpnI and SalI at 37° for 1 hr. The digested plasmid was separated and purified by the same method as above.

< Example 30 > DNA ligation and transformation

IL20GCSF-GAPDH transcription terminator digested with KpnI and SalI and YEpGalMF were ligated by T_4 DNA ligase in 30μl of ligation solution. And *E. coli* was transformed by the reacted solution. Colony

was cultured and the plasmid was purified. The plasmid was digested by restriction enzyme, and the plasmid which was inserted by IL20GCSF-GAPDH transcription terminator was selected and named as pIL20GC.

VI. Preparation of YEpHSPGC

HSP 150 (heat shock protein) is a kind of glycoprotein which is secreted from yeast under cultivation at 37°C to 42°C. By using this property of HSP 150, the expression of hGCSF can be easily controlled. The HSP 150 promoter and secretion signal were cloned first, and then YEpHSPGC was prepared by inserting hGCSF gene.

< Example 31 > PCR of HSP 150 promoter and leader sequence

The following primer was synthesized for PCR of HSP 150 promoter and leader sequence.

5′ CTA GCA GTC GAC GAT AAG TCG CCA ACT CAG CCT 3′
SalI

5′ CTA GCA GGC ACC GGC CAA AGT AGT AGC GGC CAA 3′
KpnI

About 0.4 kb of HSP 150 promoter and leader sequence was amplified by PCR from *S. cerebisiae*

genome DNA by using this primer. After purification, the plasmid was digested by KpnI and SalI.

<Example 32> Preparation of YEpHSPGC

MF(mating factor)α1 promoter and killer toxin leader sequence in YEp2kGC were substituted with HSP 150 promoter and leader sequence, and the plasmid was named as YEpHSPGC.

VII. Expression of hGCSF by using pIL20GC and YEp2kIL20GC

<Example 33> Transformation of yeast

In order to express hGCSF in yeast, yeast was transformed by pIL20GC and YEp2kIL20GC.

S. cerevisiae 2805 (a, pep4:: HIS3, pro1- , can1, GAL1, his3 , ura3-52) was inoculated to 3ml of YEPD media (1% of yeast extract, 2% of peptone, 2% of glucose), and cultured at 30°C at 250 rpm overnight. The cultured cells were reinoculated into 15ml of YEPD media. When OD600 is about 1.0 the culture was centrifuged and then competent yeast was prepared according to Alkali Cation-Yeast transform kit (Bio 101) protocol. Pellet of yeast was washed by TE buffer, suspended in lithium-acetate solution, and

cultured at 30°C at 120rpm. After centrifuging the suspended solution, the pellet was suspended in TE buffer and mixed with transformable plasmid, vector DNA, and histamine in eppendorf tube. After the mixed solution was left at room temperature for 15 minutes, PEG was added to the mixed solution and the solution was left at 30°C for 10 minutes. The reacted solution was treated by heat shock at 42°C for 5 minutes, was centrifuged, and then was suspended in 200μl of SOS media. The suspended solution was spread on SD agar plate media [0.8 g/l complete supplement Medium-URA (Bio 101), 6.7g/l Yeast Nitrogen Base without Amino Acid (DIFCO), 2% glucose, 1.5% agar], was cultured at 30°C for 3 days, and URA^+ colony was selected. Yeast transformed by pIL20GC was named as *Saccharomyces cerevisiae* GC1, yeast transformed by YEp2kIL20GC was named as *Saccharomyces cerevisiae* K2GC, and each strain was deposited to Korean Collection for Type Culture in September 27, 1995 (accession number : KCTC 0193BP and KCTC 0195BP, respectively).

<Example 34> Expression of GCSF

Colony was inoculated into SD media, and cultured at 30°C at 250rpm overnight. After the cultured solution was centrifuged, the pellet was

suspended in 1ml of YEPGal media (1% Yeast extracts, 2% peptone, 2% galactose) and cultured at 30°C at 250rpm for 15hrs in order to express hGCSF. The culture was centrifuged and 0.5 ml of the supernatant mixed with 10μg/ml BSA and 10% TCA was left on ice for 20 minutes. Then it was centrifuged at 4°C at 13,000 rpm for 10 minutes to precipitate hGCSF.

The pellet was dissolved in the solution containing 20μl of distilled water and 20μl of 2X SDS dye [125mM Tris-HCl pH 6.8, 4% of SDS, 20% of glycerol, 10% of 2-mercaptoethanol], and the solution was electrophoretically analyzed using 16% SDS(dodecyl sodium sulfate)-PAGE gel [Laemmli, Nature. 227: 680-684], and the gel was stained with Coomasie blue. As a result, 18.7 kDa band of hGCSF was visualized.

<Example 35> Production of hGCSF in fermentation culture

a) strain and medium

The hGCSF was expressed and produced in fed-batch cultures of the yeast transformed by recombinant plasmid, pIL20GC. The seed media contains 20g of glucose, 6.7g of YNB (yeast nitrogen base) without amino acid, and 0.8g of CSM-Ura (complete supplement mixture missing uracil) per liter. The

composition of media used for batch and fed-batch cultivations is as follows.

(1) Batch cultivation(per liter)

a)	KH_2PO_4	10g
	$(NH_4)_2SO4$	2g
	$CaCl_22H_2O$	0.5g
	NaCl	0.5g
	trace metal solution	10ml
	vitamin solution	1ml
	Casamino acids	5g
	Tween 80	0.6g
b)	$MgSO_4 \cdot 7H_2O$	0.5g
c)	glucose	10g

Components a), b) and c) were autoclaved separately at 121°C for 15minutes.

(2) Fed-batch cultivation

1) growth phase(per liter)

a)	$(NH_4)_2SO_4$	3g
	KH_2PO_4	5g
	vitamin solution	3.5ml
	trace metal solution	5ml
	Casamino acids	variable amounts
	Tween 80	0.6g

b) $MgSO_4 \cdot 7H_2O$ 4g

c) glucose variable amounts

The concentration ratio of sequence to casamino acids was ranged from 0.5 to 4.5 in the growth phase media.

2) Induction or product formation phase (per liter)

a) $(NH_4)_2SO_4$ 3g

KH_2PO_4 5g

vitamin solution 3.5ml

trace metal solution 5ml

Yeast extract variable amounts

Tween 80 0.6g

b) $MgSO_4 \cdot 7H_2O$ 4g

c) galactose variable amounts

The concentration ratio of glucose to yeast extract was ranged from 0.5 to 3 in the induction phase media.

Components a), b) and c) were autoclaved separately at 121°C for 15minutes.

Trace metal solution comprises per liter: 2.78g of $FeSO_4$, 1.36g of $ZnCl_2 \cdot 2H_2O$, 0.8g of $CuSO4 \cdot 5H_2O$, 2.42g of $Na_2MoO_4 \cdot 2H_2O$, 2.38g of $CoCl_2 \cdot 6H_2O$ and 1.69g of $MnSO_4$.

Vitamin solution comprises per 100mL: 0.6g of

inositol, 0.12g of Ca-pantothenate, 0.12g of pyridoxine HCl, 0.12g of thiamine and 0.01g of biotin.

b) Cultivation and hGCSF production

After recombinant yeast was cultured on the agar plate media which has the same composition as the seed media, colony was suspended in 15% glycerol solution and stored at -70°C. At the time of cultivation, the recombinant yeast stored at -70°C was spread on the above agar plate media and was cultured at 30°C, for 48hrs. Then colony was inoculated into the seed media in shake flask (250mL) and cultivated at 30°C at 250rpm. After 24hrs, this seed culture was inocluted upto 5% of batch medium and cultivated in 5L fermentor at 30°C, pH 5.5. When the glucose in the media is exhausted, the growth phase medium of fed-batch cultivation is added by pump connected to control module of fermentor. The medium feed rate was controlled with the following design equations to maintain glucose concentration below 100 mg/L.

$$\mu = \mu_0(S)\left(1 - \frac{X}{X_m}\right)$$

$$F_i = \frac{\mu X_i V_i}{S_0 Y_{x/s}}$$

$$V_{i+1} = V_i + F_i(\Delta t)$$

$$X_{i+1} V_{i+1} = X_i V_i \exp(\mu \Delta t)$$

S_0 : glucose concentration in feed media (g/L)

X : yeast concentration in culture broth (g/L)

X_m : cell mass inhibition constant (g/L)

μ : specific growth rate (hr-1)

V : culture volume (L)

F : volumetric feed rate (L/hr)

Δt : 30sec

When the concentration of yeast reached 25 to 35g/L in the growth phase, the feed medium should be switched to induction phase medium. The volumetric feed rate was controlled to maintain the concentration of galactose at 10 to 35 g/L. The dissolved oxygen concentration was maintained above 40% of air saturation in fermentation broth by controlling the agitation speed and air flow rate. Culture samples were taken from fermenter for the analysis of cell density ethanol concentration, hGCSF concentration and plasmid stability. As a result, hGCSF was produced to the concentration of 230mg/L in the fermentation broth, with suppressing ethanol accumulation and maintaining the plasmid stability above 90%.

VIII. Expression of hGCSF by using YEpHSPGC

< Example 36 > Transformation of yeast and expression of GCSF

S. cerevisiae 2805 was transformed by YEpHSPGC by using Alkali-Cation Yeast transform kit (Bio 101), and transformant was selected by Ura^+. The yeast transformed by YEpHSPGC was named as *Saccharomyces cerevisiae* HGCA, and deposited to Korean Collection for Type Culture in September 27, 1995 (accession number : KCTC 0194BP). Colony of yeast which grew on SD media without uracil was inoculated into 3ml of liquid media and incubated at 36°C and at 250rpm. Pellet precipitated after centrifugation was suspended in 1mL YEP Gal media [1% yeast extract, 2% peptone, 2% galactose] and cultivated at 37°C at 250 rpm, for 18 hr. After centrifuging the culture broth SDS dye was added to each of pellet and supernatant and protein was analyzed by 16% SDS-PAGE. In the supernatant, band of the induced recombinant protein was not visualized, butin the pellet fraction, a new major band appears at the size of 20.1kDa. Western blotting was conducted by using hGCSF Ab (R&D system) and anti-mouse IgG-alkaline phosphatase and the protein at the size of 20kDa was shown to be immunoreactive.

IX. Expression of hGCSF by using

<Example 37> Preparation of pIL20XGC

For the convenience of cloning, Xba I site was

inserted into pIL20GC.

1) PCR

At first, the oligonucleotide including XbaI site was synthesized by synthesizer (ABI, 392 DNA/RNA synthesizer) as follows.

5′ TCT CTT GTC TAG AGA AAC AGC T 3′
XbaI

The PCR was conducted by using the above primer and following primer complementary to mating factor α.

5′ ACA ATA GAG CTC TAT AAA ACA 3′
SacI

pIL20GC was used as template. 2 units Vent DNA polymerase was added to 100μl of PCR reaction solution including 200μM dNTP and 50 pmol of each primer, and then the reaction was cycled 35 times, with the following conditions:

Pretreatment 90°C, 60 seconds;
Annealing 45°C, 5 seconds;
Extension 72°C, 15 seconds;
Denaturation 94°C, 5 seconds;
Postreaction 53°C, 30 seconds

Amplified DNA of killer toxin leader sequence-IL-1ß-24-AA was saparated by electrophoresis

using 1.5% agarose gel, and DNA band of 80bp size was eluted and purified. The DNA obtained by PCR has following base sequence.

Met

ACA ATA GAG CTC TAT AAA ACA ATG AAC ATC TTC TAC ATC

TGT TAT CTC GAG ATA TTT TGT TAC TTG TAG AAG ATG TAG

Sac I

TTC TTG TTC TTG TTG TCT TTC GTT CAA GGT TTG TCA CTG

AAG AAC AAG AAC AAC AGA AAG CAA GTT CCA AAC AGT GAC

AAC TGC ACG CTC CGG GAC TCA CAG CCA AAA AGC TTG GTG

TTG ACG TGC GAG GCC CTG AGT GTC GGT TTT TCG AAC CAC

ATG TCT GGT CCA TAT GGA CTG AAA GCT GGT GTT

TAC AGA CCA GGT ATA CCT GAC TTT CGA CCA CAA

TCT CTA GAC AAG AGA

AGA GAT CTG TTC TCT

Xba I

When XbaI site is inserted by PCR, there is no change in sequence of amino acid but, there is only substitution at the level of base sequence.

2) hGCSF gene was obtained from PCR using PCR product synthesized above and the oligonucleotide complementary

to C-terminus of hGCSF gene.

The oligonucleotide complementary to C-terminus of hGCSF gene is as follows.

5′ ATG GGA GGA TCC GGG CTT GGC TCA GGG CTG GGC 3

BamH I

pIL20GC was used as template. 2 units Vent DNA polymerase was added to 100μl of reaction solution including 50pmol of primer, amplified DNA of killer toxin leader sequence-IL-1ß-24AA-XbaI-KEX2, and 20μM dNTP, and then the reaction was cycled 35 times, with the following conditions:

Pretreatment 95°C, 60 seconds;

Annealing 55°C, 5 seconds;

Extension 72°C, 15 seconds;

Denaturation 94°C, 7 seconds;

Postreaction 53°C, 30 seconds

Amplified product (killer toxin leader-IL-1ß N-terminal (24AA)-XbaI-KEX2-hGCSF) was separated by electrophoresis using 1% agarose gel, and DNA band size of 0.66 kb was eluted from gel, digested by restriction enzymes SacI and BamHI, and finally purified. 1μg of pIL20GC was digested by SacI and BamHI at 37°C for 1hr. Digested plasmid was electrophoresed in 1% agarose gel, and DNA was eluted by using Jetsorb. The PCR product

digested by SacI and BamHI and the plasmid pIL20GC were reacted in 30μl of ligation reaction solution with the addition of T_4 DNA ligase. *E. coli* XL-1 Blue was transformed with the reaction mixture. And then plasmid was purified after cultivation of colony. The plasmid which was digested by restriction enzyme, XbaI was selected and named as pIL20XGC.

<Example 38> Transformation of yeast

In order to express hGCSF in yeast, yeast was transformed by pIL20XGC. *S. cerevisiae* 2805 (a, pep4:: H153, pro 1-δ, can1, GAL1, his 3δ, ura3-52) was inoculated into 3 mL of YEPD media and cultured at 30°C at 250 rpm overnight. This was reinoculated into 15 mL of YEPD and centrifuged when OD_{600} is about 1. Then competent yeast was prepared according to Alkali Cation -Yeast transform kit (Bio 101) protocol. Pellet of yeast was washed by TE buffer, suspended in the lithium acetate solution and shaked at 30°C at 120 rpm. After centrifuging the suspension solution, the pellet was suspended in TE buffer and then was added to eppendorf tube including transformable plasmid, carrier DNA, and histamine. After kept at room temperature for 15 minutes, PEG solution was added to this solution and the resulting mixture was left at 30°C for 10 minutes.

After the mixture was heated at 42°C for 5 minutes and centrifuged, the pellet was suspended in 200μl of SOS media, spread on SD agar plate media and cultured at 30°C for 3 days. Finally URA^+ colony was selected.

This yeast strain was deposited to Korean Collection for Type Culture (accession number : KCTC 0330 BP).

<Example 39> Expression of hGCSF

The colony was inoculated into 3mL of SD media and cultured at 30°C, 250 rpm, overnight. After centrifuging culture broth, the pellet was suspended in 1ml of YEPGal medium cultured at 30°C at 250rpm for 15hrs, and finally hGCSF expression was induced. Same amount of 2X SDS dye [125mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol] was added to culture solution, and the solution was heated for 5 minutes. Proteins are separated in 15% of SDS PAGE [Laemm 1; Nature. 227: 680-684] and stained by Coomassie blue. As a result, hGCSF was expressed at the same level in the case of using pIL20GC.

X. Purification of expressed hGCSF protein

<Example 40> Ammonium sulfate precipitation

After yeast cell culture was centrifuged for 10

minutes at 10,000Xg, the supernatant was saturated at 85% with $(NH_4)_2SO_4$ and was left at 4°C for 24hrs. After centrifugation at 10,000 Xg for 30 minutes, the obtained pellet was dissolved in 50mM Tris (pH 7.8) buffer solution including 0.1mM EDTA and 1mM DTT, and insoluble substance was removed by centrifugation at 10,000xg for 10 minutes. All experiments above were conducted at 4°C. hGCSF obtained from the above process was purified further by gel-permeation chromatography.

<Example 41> Gel-permeation chromatography

The media of gel-permeation chromatography, sephacryl-S-200 (Pharmacia) was washed by 50mM Tris (pH 7.8) buffer solution including 1mM DTT and 0.1mM EDTA, and was packed into the column (1.6 × O100cm). The proteins in the 20mL culture broth was concentrated by $(NH_4)_2SO_4$ precipitiation above and the concentrated solution was loaded on cloumn and eluted by 50mM Tris (pH 7.8) buffer solution including 1mM DTT and 0.1mM EDTA. The proteins in each peak, obtained at the absorbance of 280nm was concentrated by lyophilization. According to the results of SDS-PAGE analysis of each peak sample, hGCSF and some other proteins were contained in the first peak sample, and medium

component peptides/proteins were contained in the second peak sample (Fig. 15). Most medium peptides/ proteins was removed by sephacryl-S-200 gel filtration chromatography. Partailly purified hGCSF sample by gel-permeation chromatography was subject to the next stage of C4 reversed-phase-HPLC.

<Example 42> C4 reversed-phase-HPLC

hGCSF purified partially by gel-permeation chromatography was finally purified by using C4 column reversed-phase-HPLC. C4 column used was a product of Vydac company, and column size was 1.0 × 25cm. Flow rate was 2mL/min. After about 100μg of material was injected. The column was runned with 0.1% TFA in water for 5 minutes and linear gradient in 0.1% TFA in acetonitrile was applied from 0 to 100%. The hGCSF was eluted at the gradient of 90% of 0.1% TFA in acetonitrile. By SDS-PAGE, it was confirmed that hGCSF was completely purified (Fig. 16). The recovery yield of hGCSF in the purification described in Example 44-46 above process was 8%, and about 18mg of purified hGCSF was obtained from 1L culture broth.

XI. Analysis of N-terminal amino acid of purified hGCSF

<Example 43> Analysis of N-terminal amino acid of purified hGCSF

hGCSF seperated by C4 reversed-phase-HPLC was blotted to PVDF membrane, and N-terminal amino acid sequence was determined.

The N-terminal amino acid sequence corresponds to that of mature hGCSF (NH_2-Thr-Pro-Leu-Gly-Pro-COOH). This analysis was perfomed with technical assistance of Korean Basic Science Center. Protein sequencer used in analysis is Milligen 6600B, and PTH-amino acid derivative made by Edman degradation method was analyzed by HPLC.

mobile phase A : 35mM ammonium acetate buffer (pH 4.8)

mobile phase B : 100% acetonitrile

temperature : 50°C

time (min)	flow rate (mL/min)	% A	% B	curve
INIT	0.7	95	5	*
0.7	0.7	75	25	6
1.4	0.7	73	27	6
2.8	0.7	73	27	6
5.7	0.7	55	45	6
7.4	0.7	55	45	6
8.1	0.7	52	48	6
12.0	0.7	30	70	6
20.0	0.7	95	5	6

Amino acid sequence was also determined by using polymer coupling method. 5mL of solution A was spotted on each side of membrane disk (PVDF) and the membrane disk was dried for 15 - 20 seconds. The membrane disk was put on heat board at 55°C, 30mL of solution B was spotted thereon, and dried for 7 minutes (it was never dried over 10 minutes). 5mL of solution C was spotted on each side of membrane disk, and dried for 15 - 20 seconds. And the membrane disk is put on 55°C heat board, and 30mL of solution D was spotted, and dried for 5 minutes. 20mL of solution B was spotted and dried for 5 minutes, and membrane disk was washed with ethanol, water, and methanol.

A) PITC solution (10nmol/μl of ethylacetate)

B) buffer solution (2% v/v triethylamine in 50% v/v in methanol)

C) DITC solution (0.1% w/v in ethylacetate)

D) polymer solution [0.1% w/v polyarylamine hydrochloride (low molecular weight) in B solution].

According to the result of analysis of amino acid by above method, threonine was analyzed at first cycle, prolin at the second cycle, leucine at the third cycle, glycin at the fourth cycle, and prolin at the fifth cycle. Therefore, the N-terminal amino acid

sequence of the hGCSF produced in this invention is NH_2-Thr-Pro-Leu-Gly-Pro, which corresponds to the N-terminus of authentic human hGCSF.

XII. Expression of hGH by using pIL20XGH

<Example 44> PCR of human growth hormone (hGH)

In order to conduct PCR of hGH, oligonucleotide complementary to N-terminus and C-terminus of mature human growth hormone was synthesized as follows.

5′ TGT TTC TCT AGA CAA GAG ATT CCC AAC CAT TCC CTT ATC CAG G 3′
XbaI

5′ ATG CCA GGA TCC CAG CTA GAA GCC ACA GCT GCC CTC CAC A 3′
BamHI

2 units Vent DNA polymerase is added to 100μl of reaction solution [10mM of KCl, 10mM of $(NH_4)_2SO_4$, 20mM of Tris-HCl (pH 8.8), 2mM of $MgSO_4$ 0.1% of Triton X-100] including 50 pmol of each primer and 200μM of dNTP. Using human pituitary cDNA library as a template, PCR was cycled 35 times in conditions as follows.

Pretreatment 94°C, 60 seconds;

Annealing 60°C, 5 seconds;

Extension 72°C, 10 seconds;

Denaturation 94°C, 7 seconds;

Postreaction 531°C, 30 seconds

DNA band at size of about 0.6 kb visualized on 1% agarose gel was purified and digested with restriction enzyme XbaI and BamHI. 1μg of pIL20XGC was digested with restriction enzyme, XbaI and BamHI at 37°C for 1hr, and separated in 1% of agarose gel. Then fragment of hGCSF was removed and the rest part of vector was selected and eluted. The hGH gene and pIL20XGC digested with XbaI and BamHI were ligased by T_4 DNA ligase in 30μl of ligation reaction solution. After *E. coli* XL-1 Blue was transformed by reaction mixtere, colony was cultured and then plasmid was purified. The plasmid was digested with restriction enzyme, XbaI and BamHI, and the plasmid which contains the hGH gene was selected and named as pIL20XGH.

<Example 45> Transformation of yeast

In order to express hGH in yeast, yeast was transformed by pIL20XGH. *S. cerevisiae* 2805 (a, pep4::HIS3, pro1-δ, can1, GAL1, his3δ, ura3-52) was inoculated into 3 mL of YEPD media, and cultured at 30°C at 250 rpm, overnight. Culture solution was reinoculated into 15 mL of YEPD and centrifuged at the time that OD_{600} is about 1, and then competent yeast was

prepared according to Alkali Cation-Yeast transform kit (BIO 101) protocol. Pellet of yeast was washed by TE buffer, suspended in lithium acatate solution, and shaked at 30°C at 120 rpm. The suspended solution was centrifuged, and the pellet was suspended in TE buffer and added to eppendorf tube including transformable plasmid, carrier DNA, and histamine. After the resultant was left at room temperature for 15 minutes, PEG was added to solution and left at 30°C for 10 minutes. The above solution was treated by heat shock at 42°C for 5 minutes, suspended in 200μl of SOS media, spread on the SD agar plate media, and incubated at 30°C for 3 days. Finally URA_+ colony was selected.

This strain of yeast was deposited to Korean Collection for Type Culture (accession number : KCTC 0331 BP).

<Example 46> Expression of hGH

Colony was inoculated in SD medium, and cultured at 30°C at 250 rpm overnight. Culture solution was centrifuged, and the pellet was suspensed in 1 mL of YEPGal media and cultured at 30°C at 250 rpm for 15 hrs, to induce hGH expression. 2X SDS dye [125 mM Tris-HCl, pH 6.8, 4% SDS, 20 % glycerol, 10 % 2-mercaptonethanol] was added to culture solution, and

the mixtere was heated for 5 minutes and electrophoresed by 15 % SDS PAGE (Laemmli, Nature. 227 : 680-684). The gel was stained by Coomasie blue, and hGH band corresponding to the size of about 22 kDa was detected.

<Example 47> Production of hGH in fermentation culture

(a) Strain and Medium

The hGH was produced via fed-batch cultivation of the yeast transformed by recombinant plasmid pIL20XGH. The composition of seed media is the same as that used for the hGCSF fermentation. The composition of media used in batch and fed-batch cultivations is as follows.

(1) batch cultivation(per liter)

Same as in Example 35.

(2) fed-batch cultivation

1) growth phase(per liter)

a)	$(NH_4)_2SO_4$	3g
	KH_2PO_4	5g
	vitamin solution	3.5mL
	trace metal solution	5mL
	Casamino acids	136g
	Tween 80	0.6g
b)	$MgSO_4.7H_2O$	4g

c) glucose 409g

2) induction phase or product formation phase (per liter)

a)	$(NH_4)_2SO_4$	3g
	KH_2PO_4	5g
	vitamin solution	3.5mL
	trace metal solution	5mL
	Yeast extract	167g
	Tween 80	0.6g
b)	$MgSO_4.7H_2O$	4g
c)	galactose	333g

Components a), b) and c) were autoclaved separately at 121°C for 15min.

The composition of trace metal solution and vitamin solution is same as in Example 35.

(b) Cultivation and hGH production

When the concentration of yeast reached 25 to 35g/L in the growth phase, the feed media was switched to the induction phase media above. The feed rate of media was controlled in order to maintain the concentration of galactose in the culture broth at about 18g/L. Unless otherwise mentioned, the culture storage and cultivation methods are the same as in

Example 35. As a result, with negligible ethanol accumulation, and over 80% of plasmid stability hGH concentration in the culture broth was increased to 1300mg/L.

What we claim is

1. Expression vector comprising yeast-derived promoter and yeast-derived secretion signal.

2. Expression vector of claim 1, wherein yeast derived promoter is hybrid promoter comprising GAL1-10 UAS and mating factor α-1 promoter.

3. Expression vector of claim 1, wherein yeast-derived secretion signal comprises killer toxin secretion signal and 24AA of amino terminal of IL-1ß.

4. Expression vector of claim 3, wherein killer toxin secretion signal is optimized by yeast codon usage.

5. Expression vector of claim 1, which comprises transcription terminator and GAL4 gene in addition to yeast-derived promoter and yeast derived secretion signal.

6. Expression vector of claim 5, wherein transcription terminator is transcription terminator of GAPDH.

7. Yeast expression vector YEp2-k, which comprises GAL

UAS-MFα1 promoter-killer toxin leader sequence-GAPDH transcription terminator-GAL4.

8. Expression vector YEp2kIL20GC.

9. Expression vector pIL20GC.

10. Transformant *Saccharomyces cerevisiae* K2GC (accession number : KCTC 0195 BP) which is transformed with YEp2kIL20GC.

11. Transformant *Saccharomyces cerevisiae* GC1 (accession number : KCTC 0193 BP) which is transformed with pIL20GC.

12. Expression vector which comprises promoter of heat shock protein and its secretion signal.

13. Expression vector of claim 11, wherein heat shock protein is HSP 150.

14. Expression vector YEpHSPGC.

15. Transformant *Saccharomyces cerevisiae* HGCA (accession number : KCTC 0194 BP) which is transformed

with YEpHSPGC.

16. Expression vector pIL20XGC.

17. Transformant *Saccharomyces cerevisiae* (accession number : KCTC 0330 BP) which is transformed with expression vector pIL20XGC.

18. Method of producing hGCSF by culturing transformants of claim 10, claim 11, claim 15, or claim 17.

19. Expression vector pIL20XGH.

20. Transformant *Saccharomyces cerevisiae* (accession number : KCTC 0331 BP) which is transformed with expression vector pIL20XGH.

21. Method of producing human growth hormone by culturing transformant of claim 20.

Fig. 1

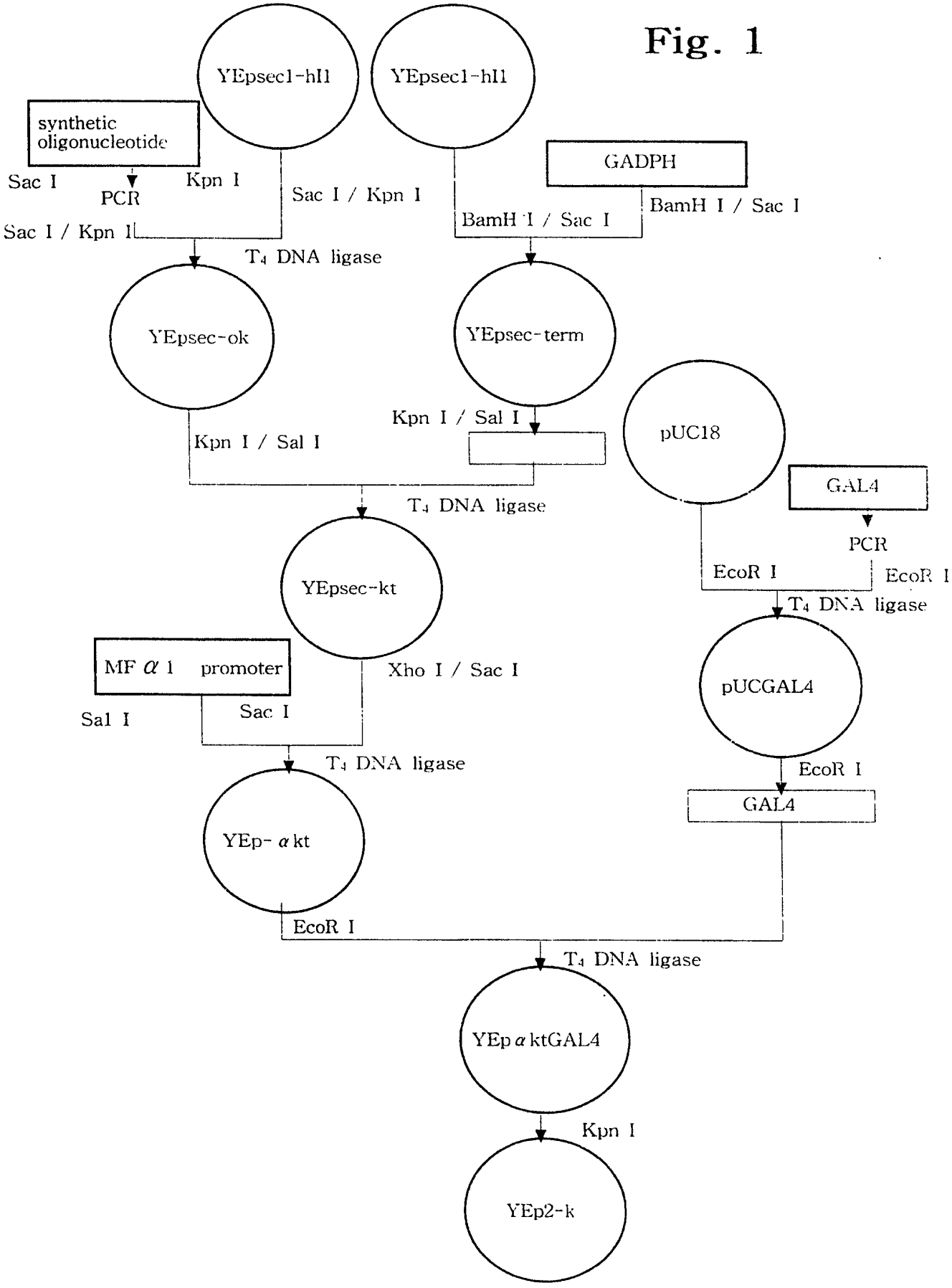

YEpsec1-hI1
YEpsec1-hI1
synthetic oligonucleotide
Sac I
PCR
Kpn I
Sac I / Kpn I
Sac I / Kpn I
GADPH
BamH I / Sac I
BamH I / Sac I
T4 DNA ligase
YEpsec-ok
YEpsec-term
Kpn I / Sal I
Kpn I / Sal I
pUC18
GAL4
PCR
EcoR I
EcoR I
T4 DNA ligase
T4 DNA ligase
YEpsec-kt
MF α 1 promoter
Xho I / Sac I
Sal I
Sac I
pUCGAL4
T4 DNA ligase
EcoR I
GAL4
YEp- α kt
EcoR I
T4 DNA ligase
YEp α ktGAL4
Kpn I
YEp2-k

Fig. 2

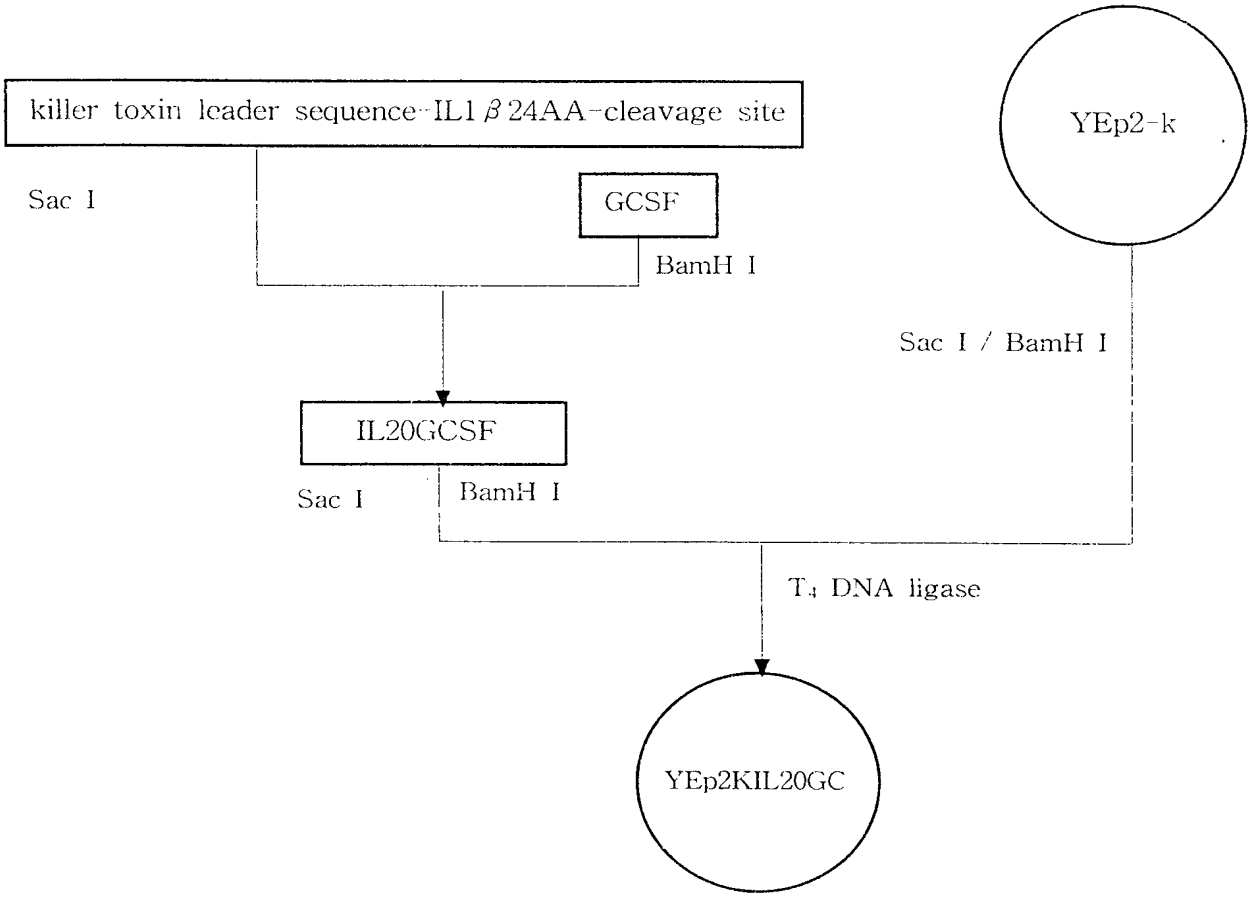
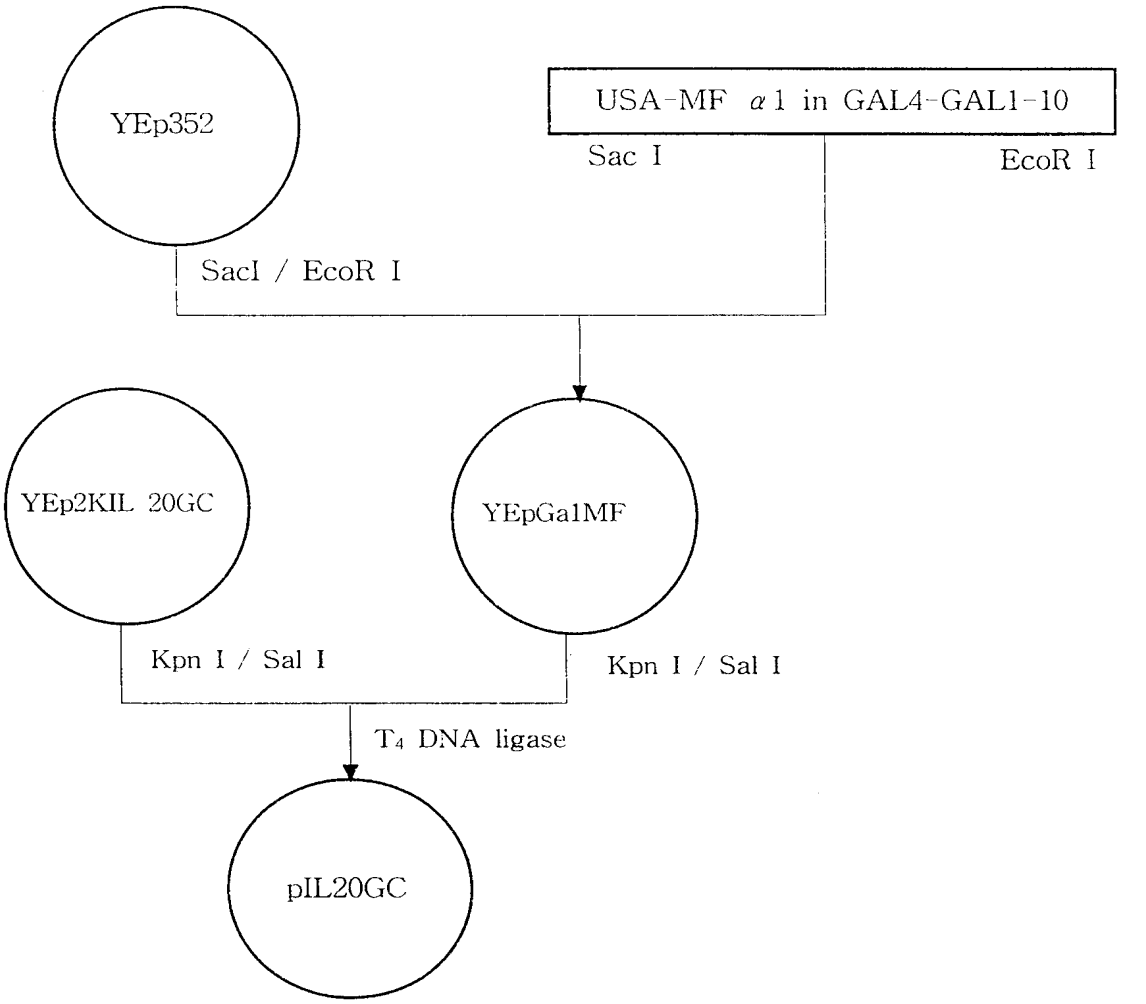

killer toxin leader sequence-IL1β24AA-cleavage site
Sac I
GCSF
BamH I
YEp2-k
Sac I / BamH I
IL20GCSF
Sac I
BamH I
T4 DNA ligase
YEp2KIL20GC

Fig. 3


YEp352
USA-MF α1 in GAL4-GAL1-10
Sac I
EcoR I
SacI / EcoR I
YEp2KIL 20GC
YEpGa1MF
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Kpn I / Sal I
T4 DNA ligase
pIL20GC

Fig. 4

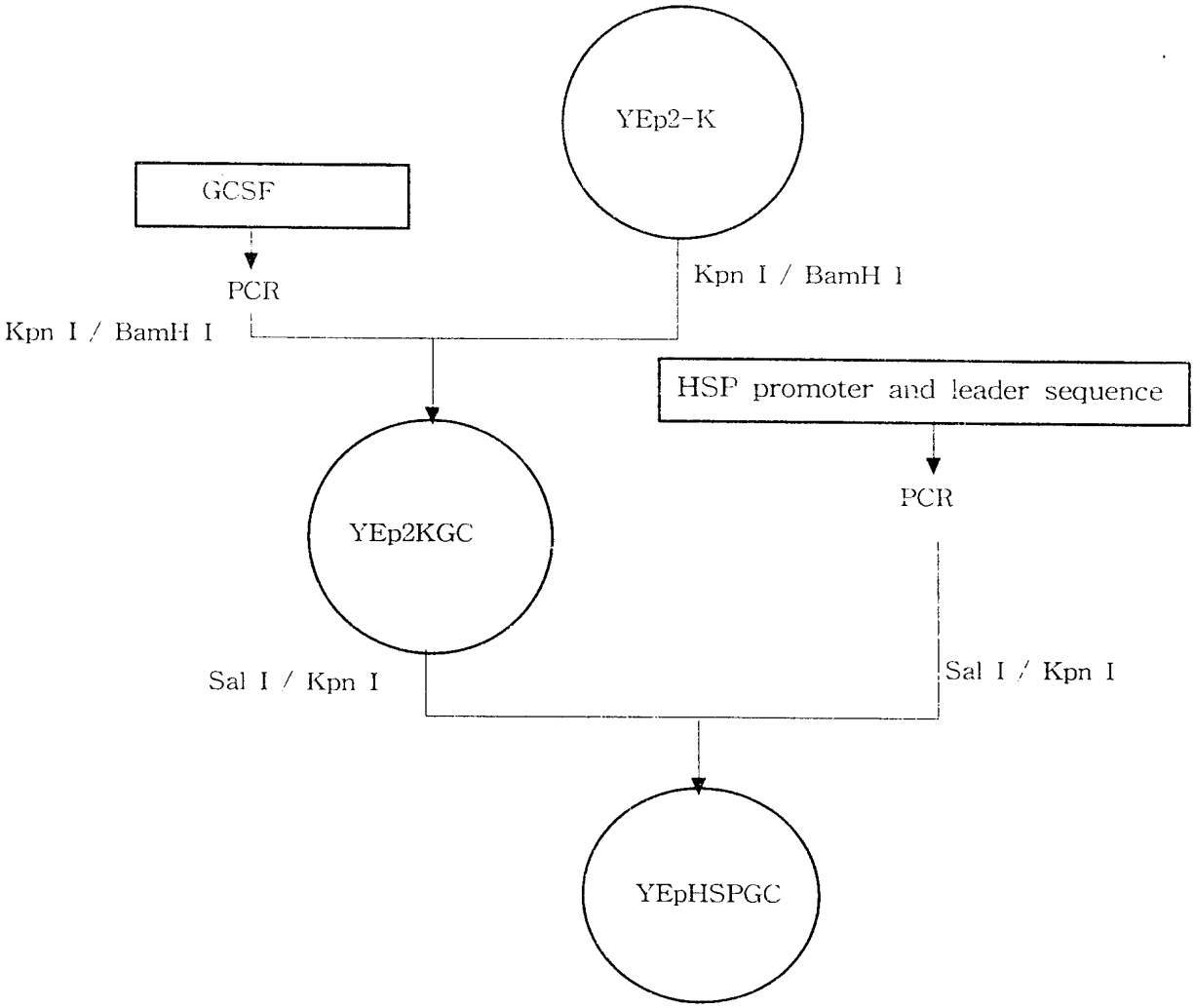

YEp2-K
GCSF
PCR
Kpn I / BamH I
Kpn I / BamH I
HSP promoter and leader sequence
PCR
YEp2KGC
Sal I / Kpn I
Sal I / Kpn I
YEpHSPGC

Fig. 5

killer toxin leader

Met-Asn-Ile-Phe-Tyr-Ile-Phe-Leu-Phe-Leu-Leu-Ser-Phe-

IL-1β

Val-Gln-Gly-Thr-Arg-Gly-Ser-Leu-Asn-Cys-Thr-Leu-Arg-

signal peptidase ↑

Asp-Ser-Gln-Gln-Lys-Ser-Leu-Val-Met-Ser-Gly-Pro-Tyr-

hGH

Glu-Leu-Lys-Ala-Gly-Val-Ser-Leu-Asp-Lys-Arg-Phe-Pro-

KEX2 ↑

Thr-Ile-Pro-Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala-Met-Leu-

Arg-Ala-His-Arg-Leu-His-Gln-Leu-Ala-Phe-Asp-Thr-Tyr-

Gln-Glu-Phe-Glu-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-

Tyr-Ser-Phe-Leu-Gln-Asn-Pro-Gln-Thr-Ser-Leu-Cys-Phe-

Ser-Glu-Ser-Ile-Pro-Thr-Pro-Ser-Asn-Arg-Glu-Glu-Thr-

Gln-Gln-Lys-Ser-Asn-Leu-Glu-Leu-Leu-Arg-Ile-Ser-Leu-

Leu-Leu-Ile-Gln-Ser-Trp-Leu-Glu-Pro-Val-Gln-Phe-Leu-

Arg-Ser-Val-Phe-Ala-Asn-Ser-Leu-Val-Tyr-Gly-Ala-Ser-

Asp-Ser-Asn-Val-Tyr-Asp-Leu-Leu-Lys-Asp-Leu-Glu-Glu-

Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg-Leu-Glu-Asp-Gly-Ser-

Pro-Arg-Thr-Gly-Gln-Ile-Phe-Lys-Gln-Thr-Tyr-Ser-Lys-

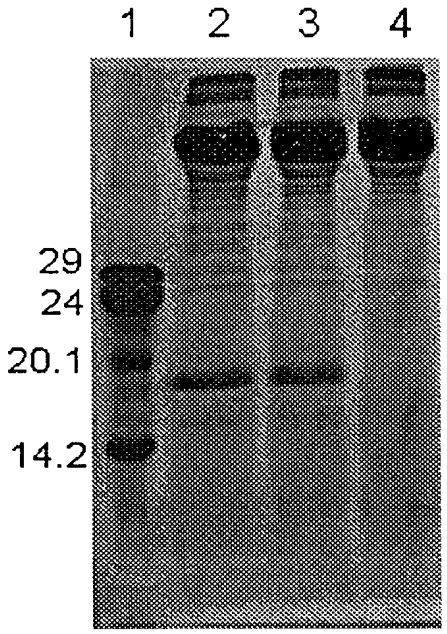
Phe-Asp-Thr-Asn-Ser-His-Asn-Asp-Asp-Ala-Leu-Leu-Lys-

Asn-Tyr-Gly-Leu-Leu-Tyr-Cys-Phe-Arg-Lys-Asp-Met-Asp-

Lys-Val-Glu-Thr-Phe-Leu-Arg-Ile-Val-Gln-Cys-Arg-Ser-

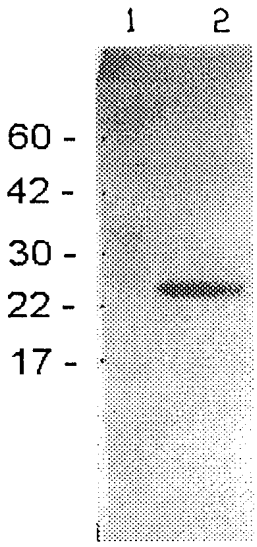
Val-Glu-Gly-Ser-Cys-Gly-Phe

Fig. 6



1 : size marker
2, 3 : pIL20GC
4: non-transformant

Fig. 7



1 : size marker
2 : YEpHSPGC

Fig. 8

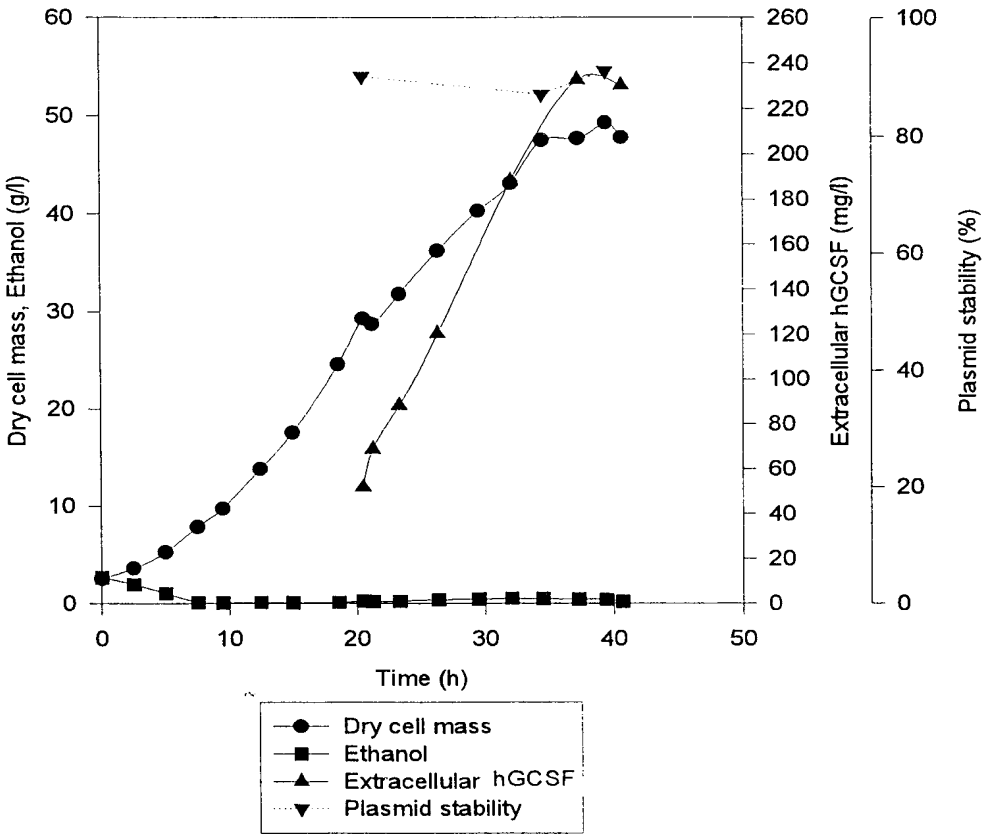
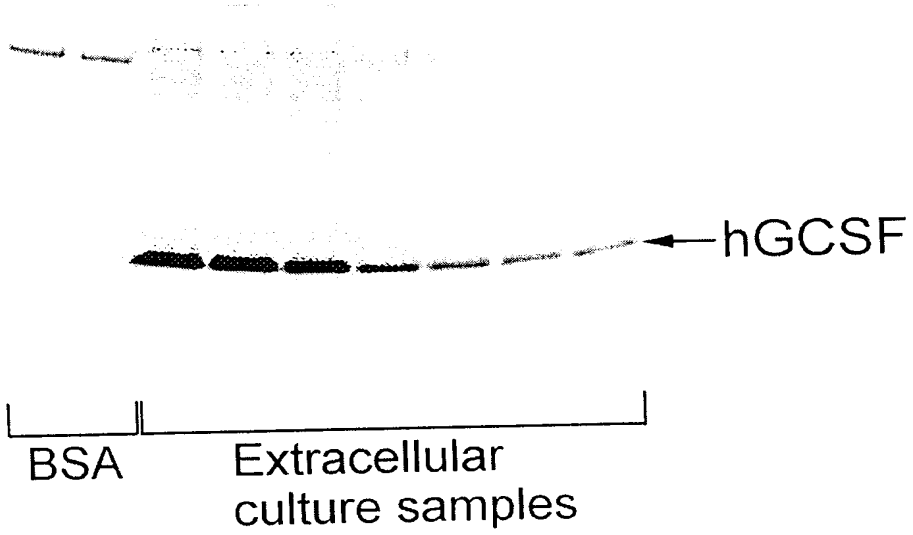

Dry cell mass, Ethanol (g/l)
Extracellular hGCSF (mg/l)
Plasmid stability (%)
Time (h)
Dry cell mass
Ethanol
Extracellular hGCSF
Plasmid stability

Fig. 9



BSA: Bovine serum albumin

Fig. 10

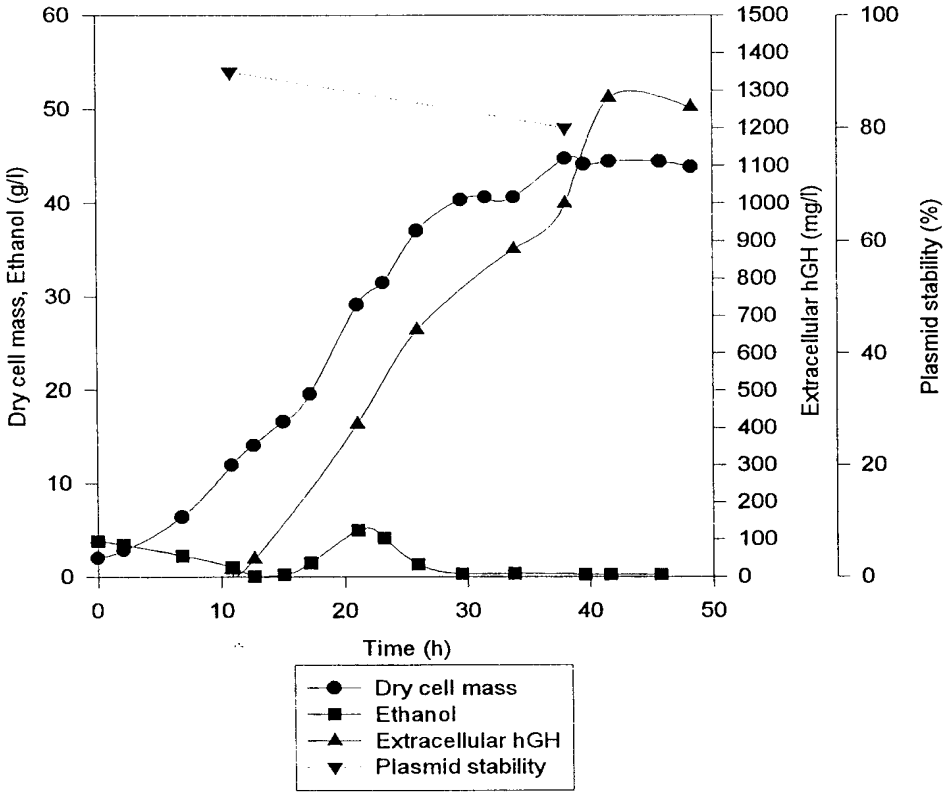
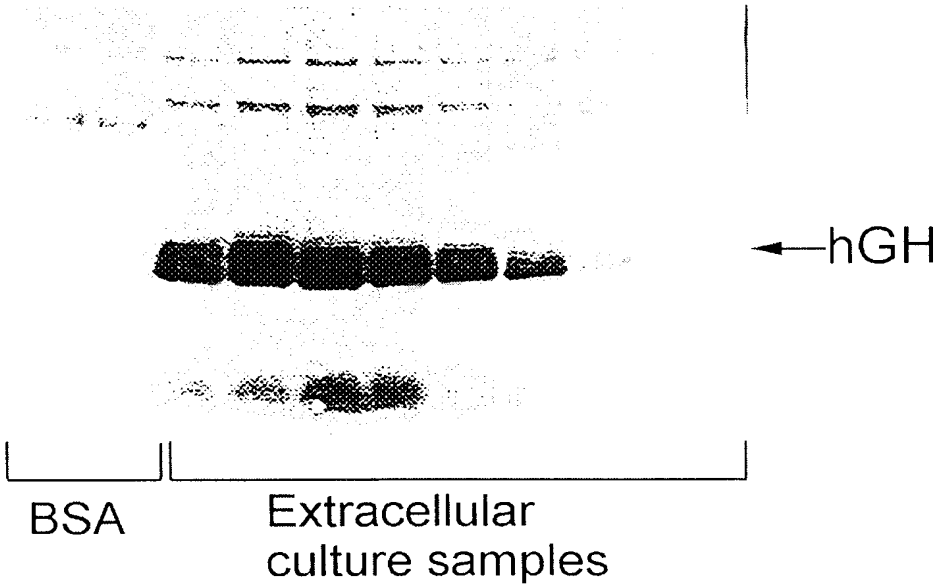

Dry cell mass, Ethanol (g/l)
Extracellular hGH (mg/l)
Plasmid stability (%)
Time (h)
Dry cell mass
Ethanol
Extracellular hGH
Plasmid stability

Fig. 11



BSA: Bovine serum albumin

Fig.12

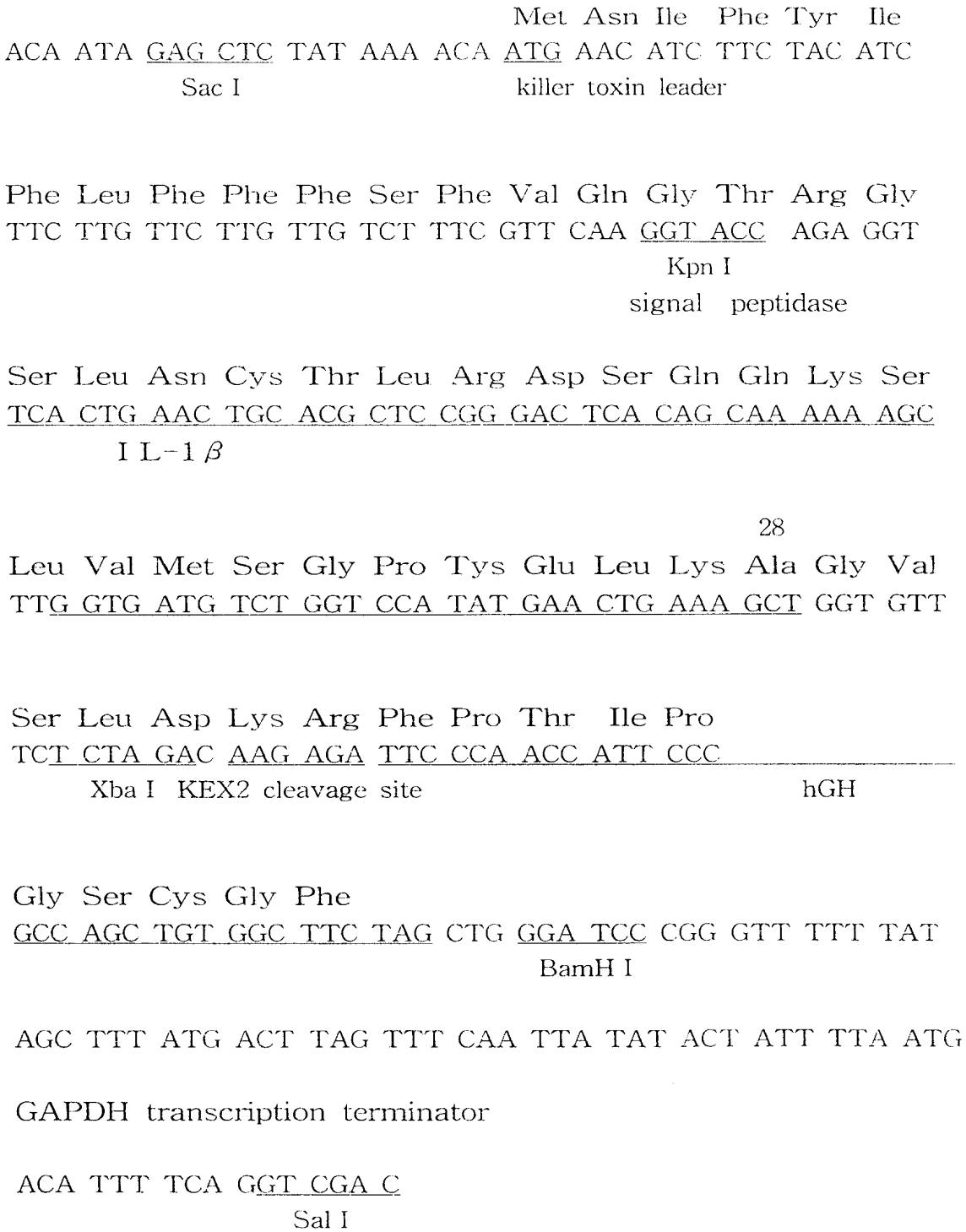

Met Asn Ile Phe Tyr Ile
ACA ATA GAG CTC TAT AAA ACA ATG AAC ATC TTC TAC ATC
Sac I
killer toxin leader
Phe Leu Phe Phe Phe Ser Phe Val Gln Gly Thr Arg Gly
TTC TTG TTC TTG TTG TCT TTC GTT CAA GGT ACC AGA GGT
Kpn I
signal peptidase
Ser Leu Asn Cys Thr Leu Arg Asp Ser Gln Gln Lys Ser
TCA CTG AAC TGC ACG CTC CGG GAC TCA CAG CAA AAA AGC
IL-1β
28
Leu Val Met Ser Gly Pro Tys Glu Leu Lys Ala Gly Val
TTG GTG ATG TCT GGT CCA TAT GAA CTG AAA GCT GGT GTT
Ser Leu Asp Lys Arg Phe Pro Thr Ile Pro
TCT CTA GAC AAG AGA TTC CCA ACC ATT CCC
Xba I KEX2 cleavage site
hGH
Gly Ser Cys Gly Phe
GCC AGC TGT GGC TTC TAG CTG GGA TCC CGG GTT TTT TAT
BamH I
AGC TTT ATG ACT TAG TTT CAA TTA TAT ACT ATT TTA ATG
GAPDH transcription terminator
ACA TTT TCA GGT CGA C
Sal I

Fig. 13

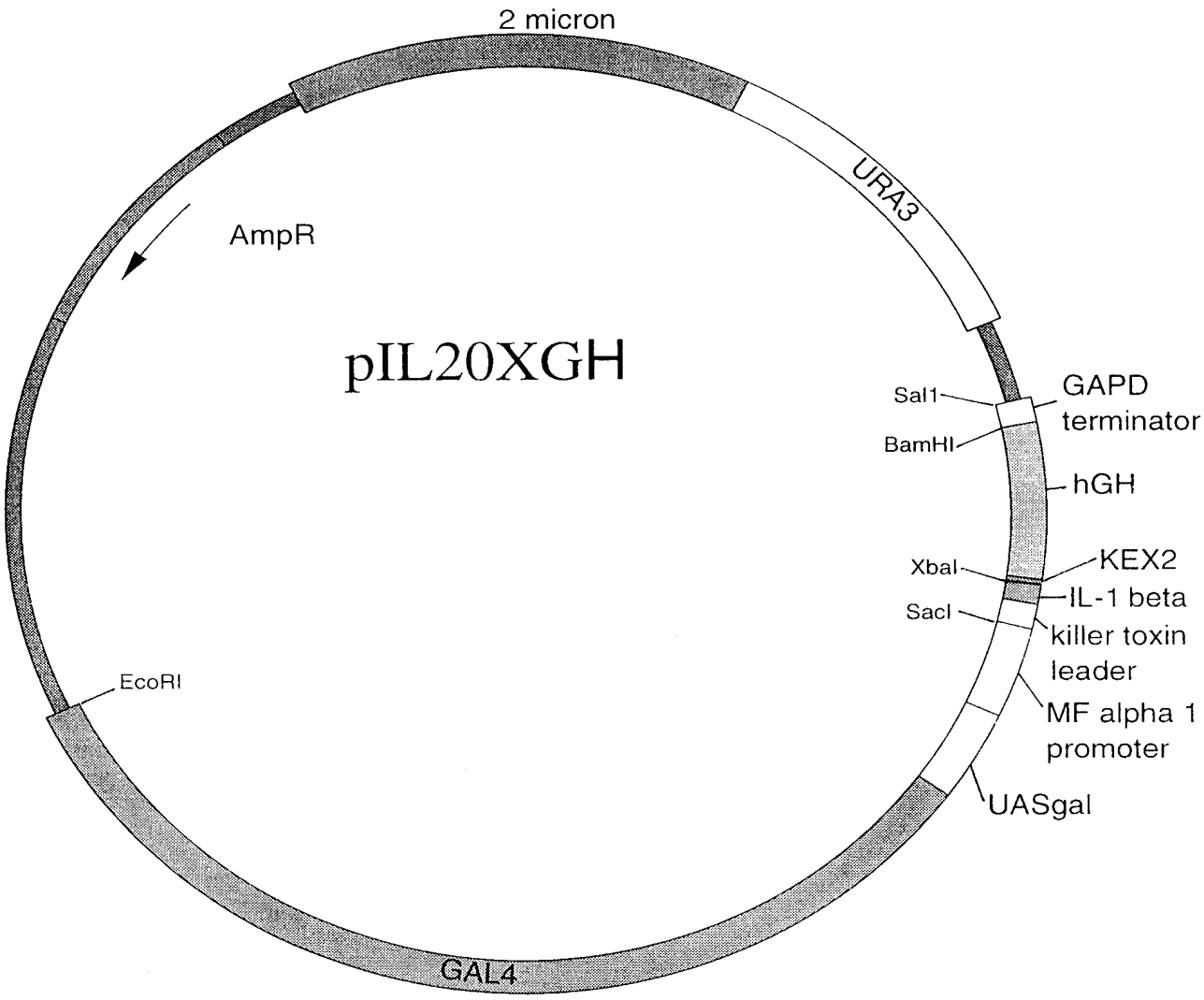

2 micron
URA3
AmpR
pIL20XGH
Sal1
GAPD
terminator
BamHI
hGH
XbaI
KEX2
IL-1 beta
SacI
killer toxin
leader
MF alpha 1
promoter
UASgal
EcoRI
GAL4

Fig. 14

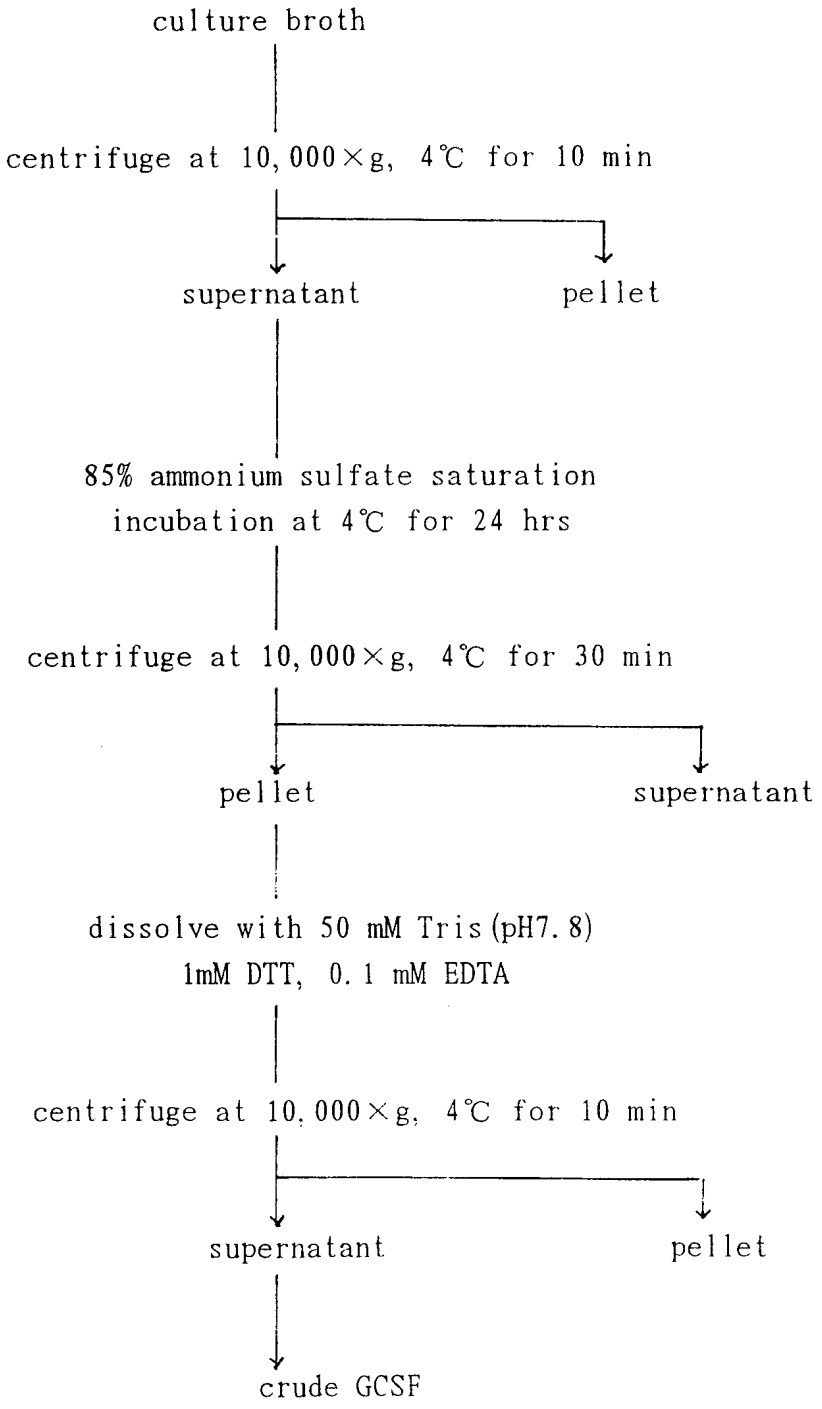
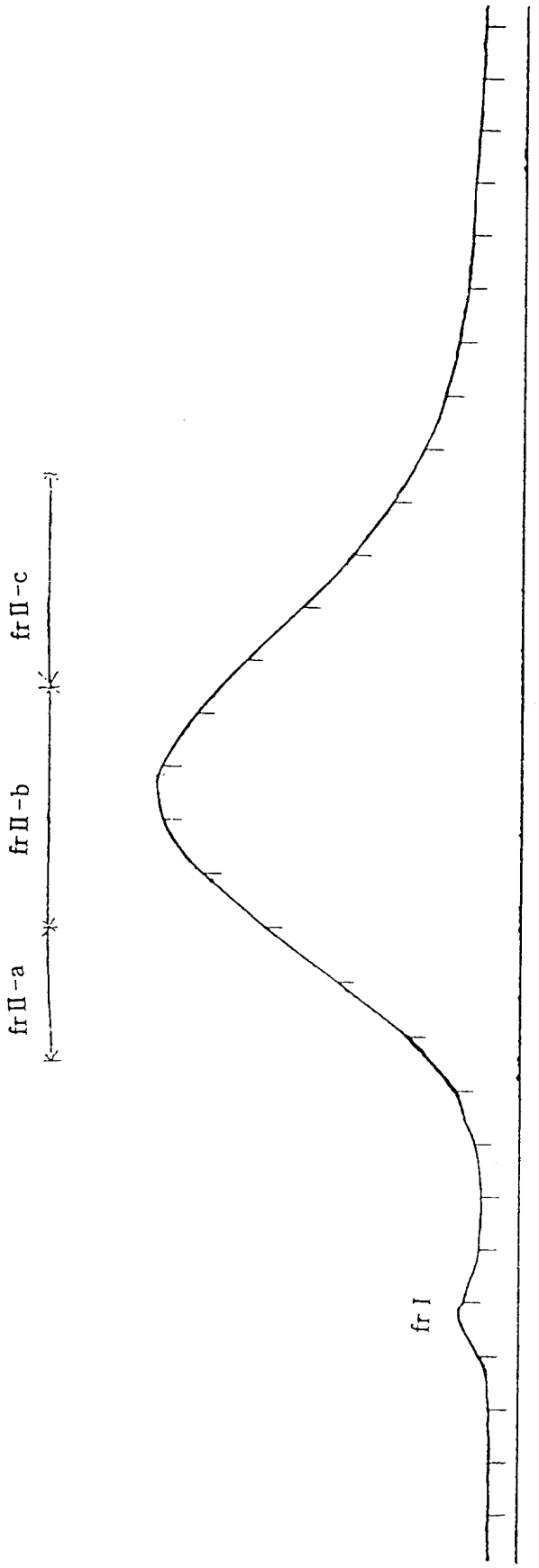

culture broth
centrifuge at 10,000×g, 4℃ for 10 min
supernatant
pellet
85% ammonium sulfate saturation
incubation at 4℃ for 24 hrs
centrifuge at 10,000×g, 4℃ for 30 min
pellet
supernatant
dissolve with 50 mM Tris(pH7.8)
1mM DTT, 0.1 mM EDTA
centrifuge at 10,000×g, 4℃ for 10 min
supernatant
pellet
crude GCSF

Fig. 15



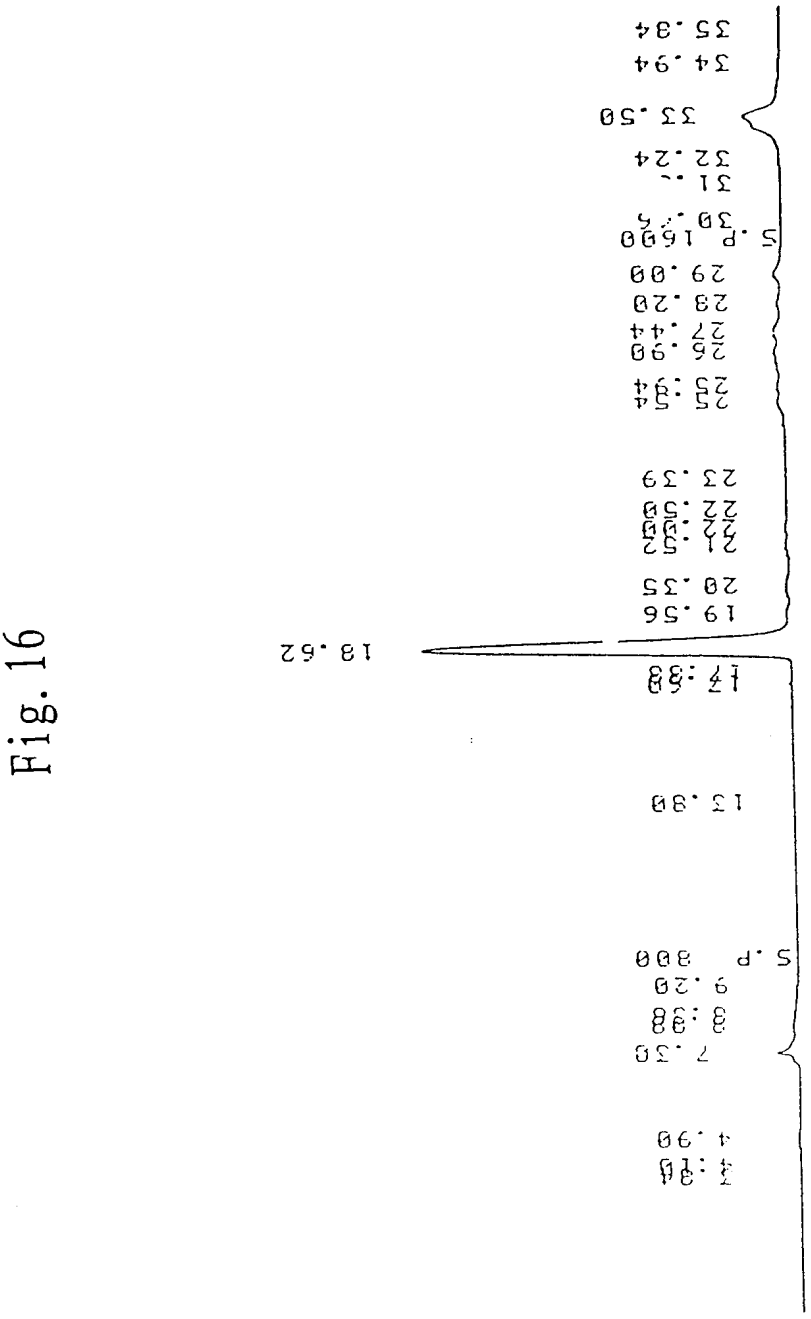

3.80
4.10
4.90
7.50
8.38
9.20
S.P 800
13.80
17.88
18.62
19.56
20.35
21.52
22.50
23.39
23.94
25.90
26.44
28.20
29.00
S.P 1600
30.76
31.--
32.24
33.50
34.94
35.84

Fig. 16

Applicant's or agent's file reference number	7 O-0402	International application No.	P ?/KR97/00097

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 42, line 17-20

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures(KCTC)

Address of depositary institution *(including postal code and country)*

KCTC, Korea Research Institute of Bioscience and Biotechnology (KRIBB)

#52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea

Date of deposit	Accession Number
September 27, 1995	KCTC 0193BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☑

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Applicant's or agent's file reference number	71)-0402	International application No.	P /KR97/00097

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 48, line 4-7.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures (KCTC)

Address of depositary institution *(including postal code and country)*

KCTC, Korea Research Institute of Bioscience and Biotechnology (KRIBB)
#52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea

Date of deposit	Accession Number
September 27, 1995	KCTC 0194BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☑

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Applicant's or agent's file reference number	7F -0402	International application No.	PCT/KR97/00097

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 42, line 17-20.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures (KCTC)

Address of depositary institution *(including postal code and country)*

KCTC, Korea Research Institute of Bioscience and Biotechnology (KRIBB)

#52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea

Date of deposit	Accession Number
September 27, 1995	KCTC 0195BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☑

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E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Applicant's or agent's file reference number	7[illegible]0-0402	International application No. P[illegible]/KR97/00097

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 53, line 5-7.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures (KCTC)

Address of depositary institution *(including postal code and country)*

KCTC, Korea Research Institute of Bioscience and Biotechnology (KRIBB)
#52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea

Date of deposit	Accession Number
May 9, 1997	KCTC 0330BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☑

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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Applicant's or agent's file reference number	?O-0402	International application No.	?CT/KR97/00097

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 60, line 14-16.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures (KCTC)

Address of depositary institution *(including postal code and country)*

KCTC, Korea Research Institute of Bioscience and Biotechnology (KRIBB)
#52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea

Date of deposit	Accession Number
May 9, 1997	KCTC 0331BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☑

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 97/00097

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 15/79, 1/19, 15/27, 15/18 // (C 12 N 1/19; C 12 R 1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 15/79, 1/19, 15/27, 15/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91/00 920 A2 (UNILEVER PLC) 24 January 1991 (24.01.91), claims 16,18,19.	1
A	EP 0 243 153 A2 (IMMUNEX CORPORATION) 28 October 1987 (28.10.87), abstract; claims 10-13; page 17, lines 5-29.	1,10,11,15,17, 18
A	EP 0 132 309 A2 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 30 January 1985 (30.01.85), abstract; pages 6-9.	1,5,20,21

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 January 1998 (13.01.98)	22 January 1998 (22.01.98)

Name and mailing address of the ISA/ AT	Authorized officer
AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna	Wolf
Facsimile No. 1/53424/535	Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 97/00097

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A2 9100920	24-01-91	AT E 160176 CA AA 2063592 DE CO 69031710 EP A1 481008 EP A1 778348 EP B1 481008 JP T2 5501949 WO A1 9100920 GB A0 8915658 CA AA 2046249 EP A1 464122 US A 5641671 JP A2 6292577 EP A1 407225 BR A 9006849 CA AA 2035043 GB A0 8915658 JP T2 4500608 US A 5658871 WO A1 9100910	15-11-97 08-01-91 18-12-97 22-04-92 11-06-97 12-11-97 15-04-93 07-03-91 03-08-89 07-01-92 08-01-92 24-06-97 21-10-94 09-01-91 27-08-91 08-01-91 03-08-89 06-02-92 19-08-97 24-01-91
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